The Effect of Stress Hormone Corticosterone on Thioredoxin System

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ABSTRACT

Background: Previous studies have shown that oxidative stress and inflammation play roles in stress-caused damage. Chronic stress is a major risk for depression and other psychiatric disorders. Studies showed that oxidative stress and inflammation can be induced by chronic stress, but inhibited by antidepressant treatment. Thioredoxin (Trx) is oxidoreductase that reverses the cysteine oxidative modifications, including cysteine sulfenylation and nitrosylation. Trx reducing activity is maintained by Trx reductase (TrxR). Trx-interacting protein (Txnip) is a negative Trx regulator. Txnip can also interact with Nod-like receptor protein 3 (NLRP3) and activate the NLRP3 inflammasome. Trx system plays an important role in regulating oxidative stress and inflammation.

Objective: The objective of this study was to investigate if chronic treatment with stress hormone corticosterone (CORT) or antidepressants regulate Trx/TrxR/Txnip expression and Trx system-regulated signaling.

Methods: HT22 mouse hippocampal cells, primary cultured mouse cerebral cortical neurons, N9 mouse microglia, and primary cultured mouse microglia were used in the present studies. Cells were treated with CORT, selective serotonin reuptake inhibitor antidepressant fluoxetine or serotonin and norepinephrine reuptake inhibitor antidepressant venlafaxine for 5 days. Protein levels of Trx, TrxR, and Txnip were measured by immunoblotting analysis. Cysteine sulfenylation was measured by dimedone conjugation method followed by immunoblotting analysis. Cysteine nitrosylation was measured by the biotin switch method followed by immunoblotting

analysis. NLRP3/Txnip binding was measured by co-immunoprecipitation. Procaspase-1 protein levels, caspase-1 activity, and IL-1β protein levels were measured by immunoblotting analysis, spectrophotometric method and enzyme-linked immunosorbent assay. Txnip knockout cells were generated by CRISPR/Cas9 technology.

Results: First, we found that although chronic CORT treatment had no effect on Trx and TrxR protein levels, this treatment significantly increased Txnip protein levels in HT22 cells and primary cultured neurons. Chronic CORT treatment increased Txnip in both nucleus and cytosol, while glucocorticoid receptor inhibitor RU486 blocked CORT-increased Txnip protein levels. Further chronic CORT treatment increased protein nitrosylation and sulfenylation while knocking out Txnip inhibited CORT-induced protein nitrosylation and sulfenylation in HT22 cells. Second, we found that chronic treatment with venlafaxine and fluoxetine increased Trx protein levels but didn't change TrxR and Txnip protein levels in HT22 cells and primary cultured neurons. Both antidepressants reversed H2O2 and CORT-induced sulfenylation, and nitric oxide donor S-nitrosoglutathione and CORT-induced nitrosylation in HT22 cells. Third, we found that chronic CORT treatment increased Txnip protein levels but didn't change Trx and TrxR protein levels in primary cultured microglia and N9 microglia. Txnip/NLRP3 protein binding was also increased in CORT-treated N9 cells, and hippocampus and frontal cortex of mouse exposed to chronic unpredictable stress. Chronic CORT treatment also decreased procaspase-1 levels, and increased caspase-1 activity and IL-1β protein levels, while Txnip knockout inhibited CORT-increased caspase-1 activity and IL-1β protein levels in N9 cells.

Conclusion: Our findings suggest that chronic CORT treatment can upregulate Txnip while chronic antidepressant treatment can upregulate Trx. CORT-increased Txnip may further inhibit Trx that promotes oxidative stress in neuronal cells, and may also increase Txnip-NLRP3 protein interaction, activate NLRP3 inflammasome, activate caspase-1 activity and releases IL-1β, which facilitate the inflammatory process. Antidepressant treatment-increased Trx may mediate the neuroprotective effect of antidepressants against oxidative stress.

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List of Abbreviations

HPA axis	Hypothalamic-pituitary-adrenal axis
CRH	Corticotropin-releasing hormone
ACTH	Adrenocorticotropin
GC	Glucocorticoids
MR	Mineralocorticoid receptors
GR	Glucocorticoid receptors
GRE	Glucocorticoid response elements
nGRE	Negative glucocorticoid response elements
NF-κB	Nuclear factor kappa B
CREB	cyclic AMP response element binding protein
IL-1β	Interleukin-1β
IFN- γ	Interferon gamma
TNF-α	Tumornecrosis factor alpha
Cox-2	Cyclooxygenase-2
CORT	Corticosterone
BDNF	Brain derived neurotrophic factor

NMDA	N-methyl-D-aspartate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
EAAT	Excitatory amino acid transporter
CNS	Central nervous system
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
GPx	Glutathione peroxidise
GSSG	Oxidized glutathione
GRd	Glutathione reductase
ETC	Electron transport chain
NADPH	Nicotinamide adenine dinucleotide phosphate
MDA	Malondialdehyde
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ARE	Antioxidant response elements

MDD	Major depressive disorder
WHO	World Health Organization
8-OHdG	8-Hydroxy-2′-deoxyguanosine
CDRS-R	Children's depression rating scale revised
NLRP3	Nod-like receptor protein 3
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin and norepinephrine reuptake inhibitor
MAOI	Monoamine oxidase inhibitor
TCA	Tricyclic antidepressant
Trx	Thioredoxin
-SOH	Sulfenic acid
-S-S-	Disulfide
$-SO_2H$	Sulfinic acid
$-SO_3H$	Sulfonic acid
-SNO	Nitrosothiols
HNO	Nitroxyl ion
ASK1	Apoptosis signal kinase-1

JNK	c-jun-N-terminal-kinase
P38	Protein 38
AP-1	Activator protein 1
Bcl2	B cell lymphoma
Bax	Bcl-2 like 1 protein
TrxR	Thioredoxin reductase
Txnip	Thioredoxin interacting protein
ASD	Autism spectrum disorder
RBANS	Repeatable Battery for the Assessment of Neuropsychological Status
DMEM	Dulbecco's Modified Eagle Media
EGTA	Ethylene glycol tetraacetic acid
DTT	Dithiothreitol
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
H_2DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
DCF	2',7'-dichlorofluorescein
NED	*N*-1-napthylethylenediamine dihydrochloride

$NaNO_2$	Sodium nitrite
MMTS	Methyl methanethiosulfonate
Biotin-HDPD	*N*-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide
NGF	Nerve growth factor
NOS	Nitric oxide synthetase
nNOS	Neuronal nitric oxide synthetase
eNOS	Endothelial nitric oxide synthetase
iNOS	Inducible nitric oxide synthetase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat domain
NACHT	Nucleotide binding and oligomerization domain
PYD	N-terminal pyrin domain
ASC	Apoptosis-associated speck like protein

CHAPTER 1: INTRODUCTION

1.1.Physiology and pathophysiology of the stress

1.1.1. Physiology of stress

Stress is a process that puts our body under a system in order to adapt environmental demand and bring physiological changes. Short-term stress as a part of our body's response to environmental threats is generally beneficial and helps the body to cope with demand. However excessive and prolonged stress can have a deleterious effect on health both physiologically and psychologically, and initiate pathophysiological processes that increase the risk of development of various psychiatric disorders[1].

The stress response is mediated mainly by the hypothalamic-pituitary-adrenal (HPA) axis in organisms. As shown in **Fig. 1**, after exposure to stress, the HPA axis is activated. Corticotropin-releasing hormone (CRH) is synthesized and released in the paraventricular nucleus of the hypothalamus. Then CRH stimulates the secretion of adrenocorticotropin (ACTH) from the anterior lobe of the pituitary. The release of ACTH into the systemic circulation leads to secretion of glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) from the adrenal cortex. The released cortisol acts as a negative feedback regulator in hypothalamus and pituitary, decreasing the secretion of CRH and ACTH respectively[2]. If the stress persists for the long time, negative feedback loop of the HPA axis can be impaired, which leads to lasting CRH hyper drive and induces high levels of GCs. Excessive GC levels can cause impairment of various organ functioning[3].

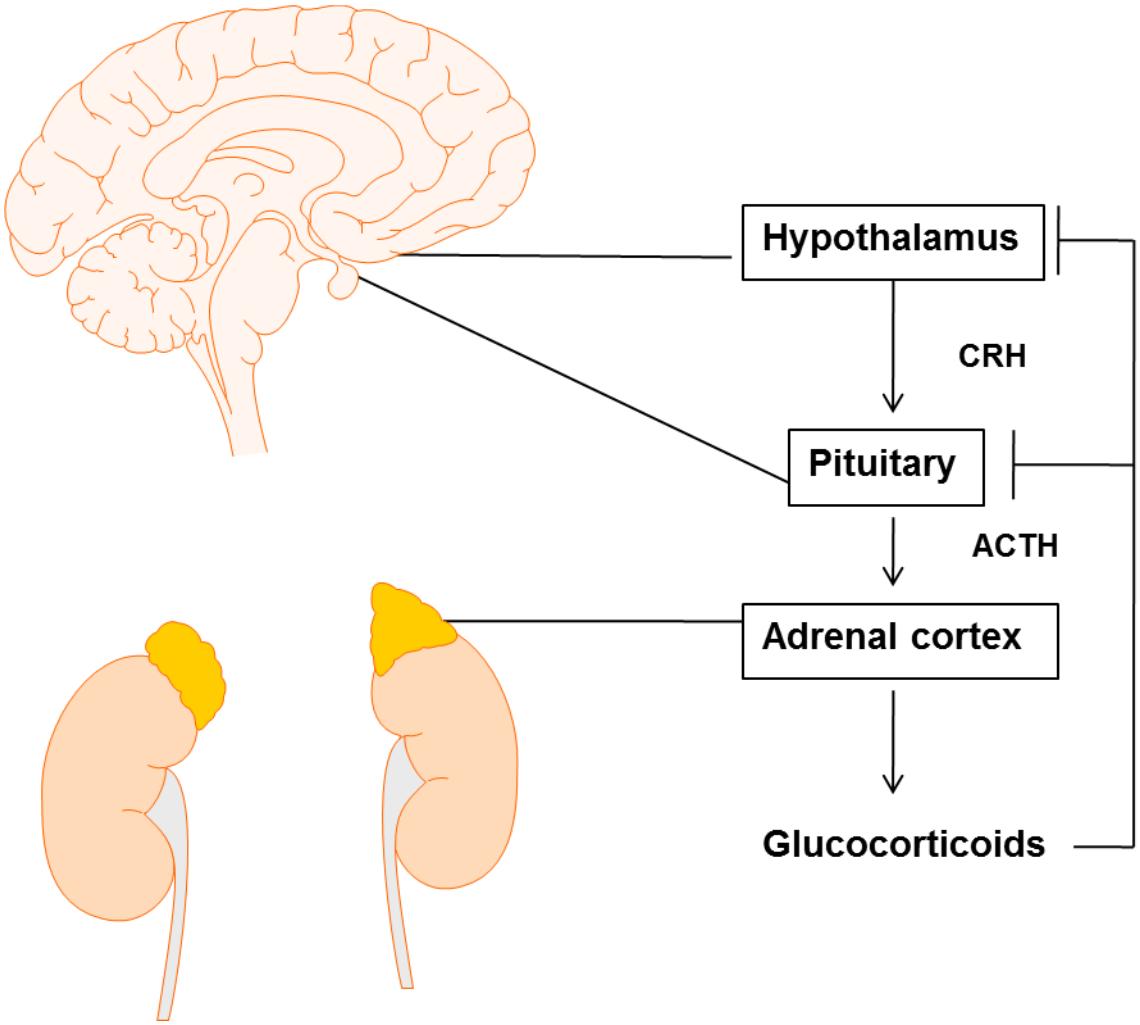


Fig. 1. Activation and feedback loop of hypothalamic-pituitary-adrenal axis (HPA). GC, glucocorticoids; CRH, corticotrophin releasing hormone; ACTH, adrenocorticotrophic hormone; →, activation; ⊥, inhibition

GCs can bind to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). GC has a high affinity for MR when compared to GR. MR is completely occupied under basal level secretion of endogenous GC during normal physiological conditions[4]. Since GR has a lower affinity for GC, higher concentrations of GC are needed for full activation of GR. In general, GR is located in the cytosol and maintained at an inactive stage by forming a multi-protein complex with molecular chaperone heat shock proteins[2]. Lipophilic GCs can easily cross the cell membrane and activate GR by three different mechanisms. As shown in **Fig. 2**, first, GCs can bind to GR and induce dissociation of GR from heat shock proteins. The dissociated GR can translocate into the nucleus and form a dimer with another GR. GR homodimer then binds to highly conserved glucocorticoid response elements (GREs) on promoter regions of many genes and activates gene expression of these targets. The response element for GR contains the consensus sequence AGAACANNNTGTTCT[5]. This process is known as transactivation. Second, GCs can also inhibit gene expression by the trans-repression mechanism. In this case, GCs bound GR can translocate into nucleus and bind to negative GRE (nGRE) sequences [CTCC (n) $_{0\text{-}2}$GGAGA] that are different from GRE sequence. The spacing required in the nGRE is variable, ranging from 0-2 nucleotides, suggesting that GR dimerization may not be necessary for nGRE-mediated trans-repression. One of the major physiological roles of nGRE mediated trans-repression process is "negative feedback" regulation of HPA axis via repression of ACTH expression. In this regulation, GCs repress the promoter of ACTH precursor pro-opiomelanocortin gene[5].

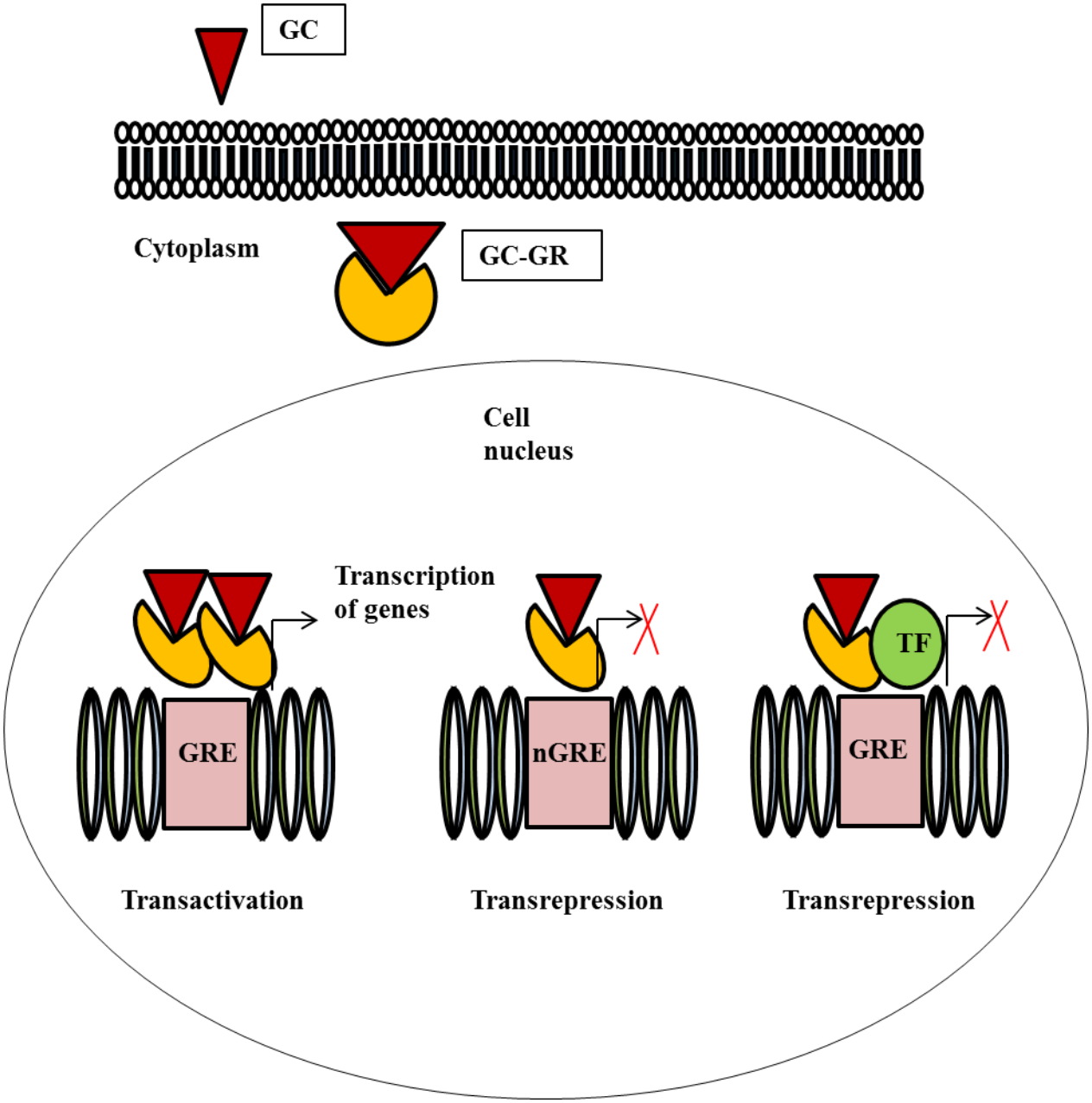


Fig. 2. Activation of glucocorticoid receptors by glucocorticoid. Transactivation (a) GC-GR dimers bind to GRE sequence and lead to transcription of genes; Transrepression (b) GC-GR complex binds to nGRE sequence Transactivation (c) GC-GR complex bind to TF and bind to GRE

Abbreviations: GC: glucocorticoids, GR: glucocorticoid receptor, TF: Transcription factor, GRE: glucocorticoid response element, nGRE: negative glucocorticoid response element

Third, GCs can also produce trans-repression action without DNA binding. In this case, GCs bound GR can translocate into nucleus and bind to other transcription factors such as nuclear factor kappa B (NF-κB), AP-1 or cyclic AMP response element binding protein (CREB)[6]. GR-mediated trans-repression gene targets include inflammatory cytokines such as interleukin-1β (IL-1β), IL-2, IL-6 and IL-12, interferon-γ (IFN-γ), tumor necrosis factor α (TNFα), cyclooxygenase-2 (COX-2) and others[7]. It has been reported that trans-repression of these genes accounts for the well-known immunosuppressive actions of GCs[7].

1.1.2. Pathophysiology of stress

1.1.2.1. Chronic stress, glucocorticoid, and impaired neuroplasticity

Many studies consistently suggest that chronic stress and excessive GC can impair neuroplasticity, cause oxidative stress and inflammation.

Neuroplasticity is the ability of the brain to reorganize neuronal connection and adapt to environmental changes. Dendritic spines of neurons are recognized to be associated with neuroplasticity and play an important role in neurotransmission[8]. Dendritic spines are small protrusions in neuronal dendrite and receive inputs from axons at excitatory synapses. In a previous study, it was found that rats exposed to restraint stress for 6 h daily for 21 days showed reduced dendritic branch points in the apical dendrites of hippocampal CA3 pyramidal neurons, and a decreased dendritic length and dendritic spine density in pyramidal neurons of the medial prefrontal cortex as compared to non-stressed rats [9]. Treatment with corticosterone (CORT) for 14 days has also shown to

decrease the hippocampal dendritic length and spine density in CA3 pyramidal neurons of rats [10]. It has also been found that administration of CORT for 3 weeks reduced the prelimbic cortical dendritic spine density in mice. GR antagonist RU-38486 inhibited CORT-reduced dendritic spine density[11]. These findings suggest that chronic stress and chronic GC exposure can impair neuroplasticity.

Brain-derived neurotrophic factor (BDNF) contributes significantly to neuronal differentiation and survival, which plays an important role in the regulation of neuroplasticity [12]. Cyclic AMP response element binding protein (CREB) is a gene transcription factor. Phosphorylated CREB can bind to the cyclic AMP response element in the promoter region of the BDNF gene, which increases BDNF gene expression [13]. Previously it has been found that total and phosphorylated CREB levels, BDNF mRNA levels, and BDNF protein levels were decreased in basolateral amygdale and hippocampus of rats exposed to chronic unpredictable stress for 3 – 5 weeks [14]. It has also been found that treatment with CORT for 21 days decreased BDNF mRNA levels in frontal cortex and hippocampus of rats [15]. GR agonist dexamethasone upon treatment for 24 hours in primary cultured mouse hippocampal neurons also decreased BDNF mRNA levels [16]. Dexamethasone-decreased BDNF mRNA levels were reversed by GR blocker RU-486[16]. Treatment with CORT for 14 days has also been found to decrease phosphorylated CREB levels and BDNF protein levels in mouse and rat hippocampus [17]. These findings suggest that chronic stress and chronic GC exposure may inhibit CREB phosphorylation, resulting in downregulation of BDNF genes. Since BDNF is known to

be a key regulator of dendritic architecture and spines [8], these studies also suggest that chronic stress may impair dendritic architecture by inhibiting BDNF signaling pathway.

Glutamate as an excitatory neurotransmitter also plays an important role in neuroplasticity. Glutamate act on the N-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor on the post-synaptic membrane. AMPA receptor is a ligand-gated Na^+ channel. Glutamate-activated AMPA receptor can increase the influx of Na^+ in post-synaptic membrane that results in depolarization. Then glutamate and co-agonist glycine bind to NMDA receptor. Activation of the NMDA receptor leads to the opening of Ca^{2+} channels. Previously chronic stress has been found to reduce levels of AMPA and NMDA receptor subunits including GluR1, GluR2, NR1, and the ratio of NR2A and NR2B, and excitatory postsynaptic potential in rodent prefrontal cortex and hippocampus[18,19], suggesting that chronic stress can decrease the NMDA and AMPA mediated synaptic currents. NMDA activation can induce Ca^{2+} currents. Glutamate membrane transporters are responsible for the removal of glutamate from the synaptic cleft. These transporters include the excitatory amino acid transporter (EAAT) 1 and 2 that are present on the membranes of astrocyte, EAAT3 and 4 that are present on neurons and EAAT5 that is present on the retina. Low levels of EAAT2 are also present on hippocampal CA3 pyramidal neurons. EAAT 2 is responsible for over 90% of glutamate uptake within the central nervous system (CNS)[20] . Previously chronic stress and CORT treatment were found to reduce EAAT2 protein levels and [^{3}H] glutamate uptake in rat hippocampus[21–23], suggesting that

chronic stress decrease glutamate transporter expression that leads to impairment in the function of glutamate uptake process.

Taken together, chronic stress and excessive GC exposure may induce abnormal neuroplasticity by reducing the dendritic length and dendritic spine density, impairing neurotrophic signaling and downregulating glutamate neurotransmission.

1.1.2.2. Chronic stress, glucocorticoid, and oxidative stress

Oxidative stress is caused by reactive oxygen species (ROS). ROS are highly reactive molecules with partially reduced oxygen that are produced as by-products of cellular metabolism. ROS include superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals. ROS can be scavenged by the antioxidant defense system. For example, superoxide can be dismutated to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is then converted to water by catalase (CAT) enzyme. Active glutathione (GSH) at a reduced state can also reduce H_2O_2 to water. This reaction is catalyzed by glutathione peroxidase (GPx). Oxidized GSH (GSSG) can be reduced back to active reduced GSH by glutathione reductase (GRd). Oxidative stress is defined as an imbalance between the production of ROS and the ability of the endogenous antioxidant defensive system in the body to produce antioxidants [24]. When ROS levels are high either resulting from ROS overproduction or deficiency of antioxidant defense, ROS become detrimental and attack many molecules of cellular machinery including DNA, proteins, and lipids, leading to their structural as well as functional damage[25]. Previously it was found that mice exposed to chronic restraint stress for 8 weeks increased ROS levels in frontal cortex and hippocampus[26].

Chronic restraint stress for 2 weeks was also found to increase superoxide levels in mouse hippocampus[27]. It has been found that GR agonist dexamethasone increased ROS levels in hippocampal slice[28]. CORT treatment was also found to increase ROS levels in HT22 mouse hippocampal cells[27]. These studies indicate that chronic stress and GC treatment increase ROS production.

Mitochondria are the main source for ROS production. Impaired mitochondrial electron transport chain (ETC) can leak electrons that can induce oxygen partial reduction and increase ROS production. Rats exposed to restraint stress for 14 and 21 days showed decreased activities of mitochondrial ETC complex I and III in forebrain as compared to controls [29] . It was also reported that rats exposed to chronic unpredictable stress for 40 days showed decreased ETC complex I, III and IV activities in the cerebral cortex as compared to controls [30]. Our laboratory has also found that treatment with CORT for 24 h decreased mitochondrial ETC complex I activity in cultured rat phaeochromocytoma PC12 cells [31] . These studies suggest that chronic stress and CORT treatment can impair mitochondrial functioning, which may induce ROS overproduction.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme complex. Activation of NADPH oxidase produces superoxide radicals. Two cytosolic regulatory subunits including p47 phox and p67 phox are important in the activation of NADPH oxidase. It has been found that mRNA levels of p47 phox and p67 phox were increased in the hippocampus and frontal cortex of mice exposed to chronic restraint stress for 14 days when compared to controls [27]. Protein and mRNA levels of

p47 phox subunit of NADPH oxidase were also found to be increased in nucleus accumbens and frontal cortex of mice exposed to social isolation stress for 2, 4 and 7 weeks[32]. Treatment with GR agonist dexamethasone for 4 h was also found to increase mRNA levels of NADPH oxidase p47 phox subunit in cultured hippocampal slices [28]. CORT treatment for 24h also increased the mRNA levels of p47phox and p67phox in SH-SY5Y human neuroblastoma cells[27]. Knocking out p47phox inhibited CORT-induced production of ROS in these cells[33]. These studies suggest that both chronic stress and GC treatment not only impair mitochondrial function but also upregulate NADPH oxidase, subsequently resulting in ROS overproduction.

Brain cells have a high metabolic rate, abundant polyunsaturated fatty acids and modest concentration of antioxidants that make the brain more susceptible to attack by ROS. Lipid peroxidation can generate malondialdehyde (MDA) and lead to altered activity of membrane receptors, decreased activity of membrane-bound enzyme and altered permeability of membrane [34]. Protein oxidation can generate carbonyl groups in residues of proline, arginine, histidine and lysine, and lead to protein misfolding, resulting in cellular dysfunction [35]. Exposure of rats to chronic unpredictable stress for 40 days not only showed increased MDA levels but also showed decreased GSH/GSSG ratio, GPx activity and GRd activity in prefrontal cortex[36]. Studies have also shown that restraint stress for 21 days increased MDA levels and protein carbonyl groups, but decreased levels of SOD, GSH, GPx, GRd and CAT in rat brain [37,38]. It is interesting that treatment with CORT for 21 days also increased MDA levels and protein carbonyl groups, and decreased levels of these antioxidant enzymes in rat brain [37]. Chronic treatment with

CORT for 14 days was also found to increase protein carbonyls and lipid hydroperoxides and decreased SOD, CAT and GPx activities in rat hippocampus[39]. Previously our laboratory found that CORT treatment for 24 h increased protein carbonylation in rat PC12 cells [31]. Our laboratory also found chronic unpredictable stress not only increased MDA levels but also decreased total antioxidant capacity, GPx activity and CAT activity in rat frontal cortex and hippocampus. These findings together suggest that chronic stress and GC treatment may induce oxidative stress by inducing both ROS overproduction and antioxidant defense deficiency. Nrf2, as a transcription factor, can form a heterodimer with small Maf protein that binds to antioxidant response elements (ARE) of promoter regions in various antioxidant genes including GPx, GRd, CAT, and SOD. Binding of Nrf2 to ARE sites can increase the expression of these genes. It has been reported that both chronic unpredictable stress and chronic restraint stress decreased mRNA and protein levels of Nrf2 in rat hippocampus[40,41]. Chronic treatment with CORT was also found to decreased Nrf2 protein levels in mouse cerebral cortex[42]. These studies suggest that chronic stress and GC treatment may decrease antioxidant enzyme levels by targeting Nrf2.

1.1.2.3. Chronic stress, glucocorticoid, and inflammation

Inflammation is an immune response of body tissues to pathogens, damaged cells, and other toxins. Cytokines are small secreted proteins released by activated macrophages, lymphocytes, mast cells, and microglia, and mediate the inflammatory response. Released cytokines, in turn, lead to the production of chemokines that are secondary inflammatory mediators.

Chronic stress and excessive GC can increase the expression and release of pro-inflammatory cytokines and chemokines. It has been found that mice exposed to chronic unpredictable stress for 3 weeks exhibited increased levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β in frontal cortex and hippocampus[43]. Chronic unpredictable stress was also found to increase IL-1β and TNF-α in both hippocampus and frontal cortex of rats[44]. The increased levels of these pro-inflammatory cytokines were reduced by GR antagonist RU-486[44], suggesting that chronic stress increases the inflammatory process via GR. It has also been reported that treatment with CORT for 3 days increased endotoxin lipopolysaccharide (LPS)-induced pro-inflammatory cytokines TNF-α and IL-1β in both frontal cortex and hippocampus of rats[45]. This treatment also increased mRNA levels of IL-1β in the hippocampus of rats[45]. These findings suggest that both chronic stress and GC increase the mRNA and protein levels of pro-inflammatory cytokines.

1.2. Chronic stress as a risk factor for major depressive disorder

Major depressive disorder (MDD) is a mental disorder that is characterized by persistent low mood, low self-esteem, low energy and loss of interest in normally enjoyable activities. A survey conducted by Statistics Canada in 2012 reported that 5.4% of the people aged 15 years or over met the criteria for mood disorders including 4.7% for major depression and 1.5% for bipolar disorder in Canada[46]. According to the World Health Organization (WHO), depression accounts for the 4th leading cause of disability worldwide [47]. It has been speculated that by 2020, depression will be ranked 2nd among leading causes of disability[48]. Nearly one in four women and one in six men may

experience depression in their lifetime[49]. WHO has estimated the costs for psychosocial counseling and antidepressant medications from 2016-2030 amounted to US$ 147 billion worldwide[50]. Chronic stress can initiate pathophysiological processes that increase the risk of development of depression. Previous studies have indicated that certain stressful major life events such as social rejection, disruption to person's goals, termination of close confidant, financial loss, and major health-related events are strongly related to increased prevalence of depression[51,52]. Both clinical and preclinical studies indicate that chronic stress or chronic treatment with stress hormones in animals can change behaviors to depressive state [53,54] .

1.3. Oxidative stress and inflammation in major depressive disorder

In spite of many years of research, the mechanisms of depression development are not clearly understood. An early theory is that monoamine dysfunction causes depression, is based on increased monoamine by antidepressants. However, these drugs increase synaptic monoamines within only minutes but often take 2-4 weeks to produce clinical response [55] , suggesting that additional factors are crucial for their therapeutic effects.

Increasing piece of evidence suggests that oxidative stress plays an important role in the underlying pathology of MDD. The findings entail an increase in biomarkers of oxidative stress but also a decrease in levels of antioxidant molecules necessary for maintaining redox homeostasis in MDD. Previously our laboratory reported that oxidation of RNA was increased in postmortem hippocampal samples of patients with MDD and bipolar disorder as compared to controls [56]. Glutathione is a major antioxidant in the brain. Our

laboratory also found that glutathione levels were decreased in postmortem frontal cortex of patients with MDD [57]. Oxidative damage has also been found in peripheral blood samples of patients. A clinical study has shown that protein carbonyl content was increased in the serum of depressed patients when compared to healthy controls [58] . Protein carbonyls were also found to be higher in the plasma samples of patients with MDD [59]. In a post-stroke depression study, it was found that protein carbonyl and 3-nitrotyrosine levels were increased, while thiol levels and catalase activity were decreased in the plasma of these stroke patients as compared to healthy controls[60]. Carbonyl group can be generated by oxidative damage to protein in side chains of lysine, arginine, proline and threonine residues [61,62] , increased carbonyls suggest that protein oxidation is associated with depression.

MDA represents an end product of polyunsaturated fatty acid and arachidonic acid peroxidation. MDA is a reactive aldehyde and forms covalent protein adducts known as advanced lipid oxidation end products. MDA also reacts with deoxyguanosine and forms DNA adducts. Therefore MDA can further damage protein and DNA. It was found that MDA levels were increased in serum of patients with MDD as compared to controls [63] . A meta-analysis and systematic review study summarized 10 studies of DNA oxidation and 8 studies of lipid peroxidation in MDD patients[64]. This study concluded that in the patients with MDD, there is increased level of DNA oxidation marker 8-Hydroxy-2′-deoxyguanosine (8-OHdG) and lipid oxidation marker F2-isoprostanes, indicating lipid peroxidation and DNA oxidation are also associated with MDD. Endogenous antioxidants are also decreased in patients with MDD. For example, it was reported that

levels of small molecule antioxidants such as vitamin E, vitamin C, CoQ, folate, and vitamin B12 are decreased in plasma, blood and serum of patients with MDD[65–68]. Serum SOD level and red blood cell SOD activity were also decreased in serum and red blood cells of MDD patients [69,70]. GPx and CAT activities were also lowered in MDD patients as compared to healthy individuals[71,72]. Taken together, these studies suggest that oxidative damage is increased in MDD patients. This increase may result from a significant imbalance between increased ROS production and deficiency of antioxidants.

Pro-inflammatory cytokines are also increased in MDD patients. Studies have shown that IL-6 and TNF-α levels were increased in plasma and cerebrospinal fluid of patients with MDD as compared to controls [73,74]. NF-κB is a transcription factor that can be activated by cytokines, free radicals, stress, and antigens, and plays an important role in the regulation of inflammatory process [75]. It has been found that cytokine TNF-α-induced NF- κB activation was higher in monocytes of both BD and MDD patients than in monocytes of controls[76]. A correlation study was performed to understand the relationship between NF- κB activation in monocytes and severity of depressive symptoms by using children's depression rating scale-revised (CDRS-R). It was found that higher levels of stimulated NF- κB in monocytes were associated with higher CDRS-R score [76], suggesting that NF- κB activation is associated with increased severity of depression. IL-6 levels in serum and NF- κB DNA binding activity in peripheral blood mononuclear cells were also found to be increased in depressed male patients subjected to trier social stress test[77]. Nod-like receptor protein 3 (NLRP3) inflammasome regulates immune response upon stimulation. Activation of NLRP3 inflammasome releases pro-

inflammatory cytokines IL-18 and IL-1β. It has been found that NLRP3 mRNA levels, and the protein levels of IL-1β and IL-18 were increased in mononuclear cells of patients with MDD when compared to controls[78]. These studies indicate that inflammation may contribute to depression development.

1.4. Protective effects of antidepressants against oxidative stress and inflammation

Antidepressants are the medications that are used to treat MDD. These drugs can be classified into four major groups including selective serotonin reuptake inhibitor (SSRI), serotonin and norepinephrine reuptake inhibitor (SNRI), monoamine oxidase inhibitor (MAOI) and tricyclic antidepressants (TCA).

Much evidence indicates that antidepressants inhibit oxidative stress and inflammation. It has been reported that chronic restraint stress inhibited activities of SOD, catalase, glutathione, and glutathione reductase, while chronic treatment with SSRI fluoxetine, TCA imipramine and SNRI venlafaxine for 21 days can reversed this inhibition in mouse hippocampus [79]. Chronic treatment with venlafaxine also showed to inhibit chronic restrain stress-increased MDA and 8-OHdG levels in mouse hippocampus [80]. It has also been found that treatment with SSRIs such as fluoxetine, fluvoxamine, paroxetine, and sertraline for 24 hours inhibited LPS-increased TNF-α and nitric oxide production in BV2 microglia cell line [81] . Clinical studies also supported that antidepressants may inhibit oxidative stress and inflammation. It has been found that MDA levels were increased in erythrocyte and plasma of MDD patients, while treatment with SSRI fluoxetine, sertraline, fluvoxamine, and citalopram for 12 weeks restored MDA levels[82].

Serum MDA levels were increased in MDD patients but decreased by treatment with fluoxetine and citalopram for 4 weeks in these patients [83]. The plasma levels of cytokines IL-2, IL-4, IL-6, IL-10 and IFN-γ were found to be increased in MDD patients, while treatment with sertraline for 6 weeks decreased the levels of these cytokines [84]. These studies suggest that oxidative stress and inflammation processes may be targeted by the pharmacological treatment with antidepressants.

1.5. Thioredoxin antioxidant system

1.5.1. Thioredoxin

Thioredoxin (Trx) is a small 12 kD oxidoreductase protein present in all living cells and produces a protective effect against oxidative stress [85]. It includes two major isoforms: cytosolic Trx1 and mitochondrial Trx2. Trx has a redox-active dithiol in the active site that contains the highly conserved sequence: -Trp-Cys^{32}-Gly-Pro-Cys^{35}-Lys-. The thiol groups in cysteine residues play an important role in defense against oxidative stress by thiol-disulfide exchange mechanism. Trx in the reduced form reduces the oxidized protein by thiol-disulfide exchange mechanism. During this process, the active site thiols of Trx get oxidized. Thioredoxin reductase (TrxR) takes electrons from NADPH and transfers to the oxidized Trx that further reduces the Trx. **(Fig. 3)**, Apart from the two cysteine residues present in the catalytic site, there are three more cysteine residues at position 62, 69 and 73. These cysteines are important for various post-translational modifications such as thiol oxidation and glutathionylation.

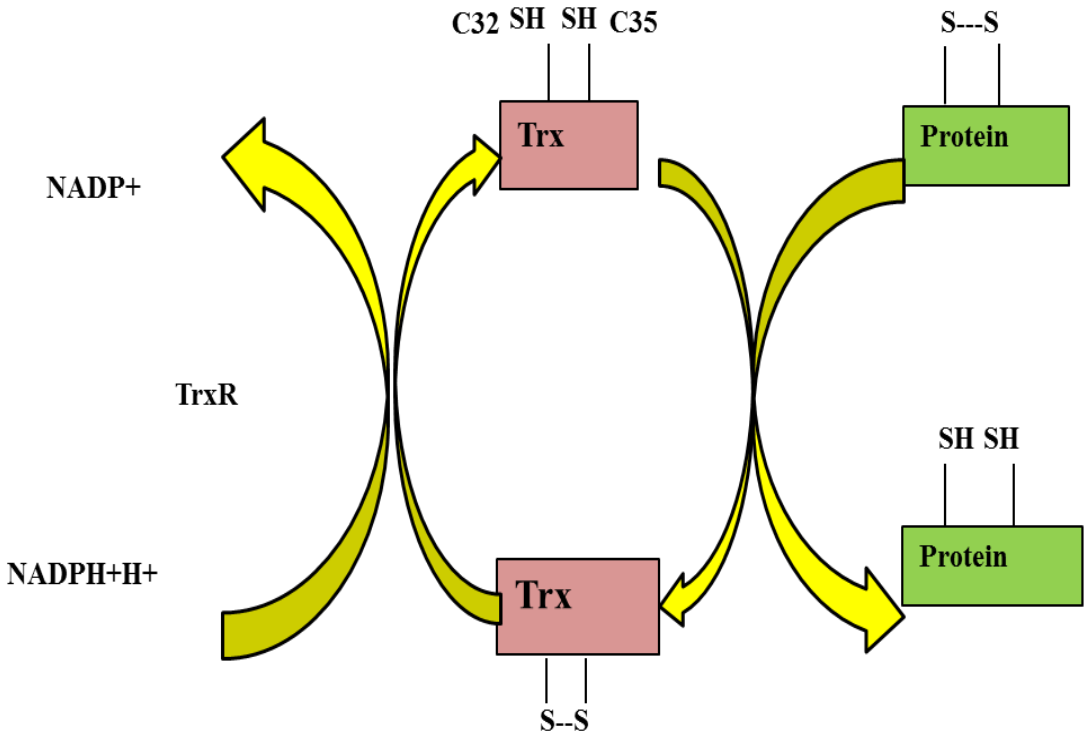


Fig. 3. Regulation of Thioredoxin system: Reduced Trx can reduce oxidized protein by thiol disulfide exchange mechanism. During this, reduced Trx gets oxidized and gets reduced by the help of TrxR using NADPH. Txnip act as negative regulator of Trx.

Abbreviations: Trx: Thioredoxin, TrxR: Thioredoxin reductase

Trx and cysteine oxidative modification: Trx can inhibit oxidative stress by reducing protein cysteine oxidation and nitrosylation. Cysteine residues in proteins are critical for regulating functions of many proteins such as redox homeostasis, metal binding, enzymatic catalysis and signal transduction [86]. The highly nucleophilic character of cysteines makes them more susceptible to ROS attack as compared to other amino acids. In the condition of oxidative and nitrosative stress, cysteine thiols of various proteins are oxidatively modified. These thiol modifications can be both reversible and irreversible. As shown in **Fig. 4,** increased H_2O_2 can attack cysteine and lead to the formation of sulfenic acid (-SOH) (sulfenylation). This protein oxidative modification is reversible. Sulfenic acid can react with thiols to form disulfides (-S-S-) (disulfidation). Trx can reduce these disulfides back to cysteine thiols via thiol-disulfide exchange mechanism. Oxidation of sulfenic acid to higher oxidation states generates sulfinic acid ($-SO_2H$) (sulfinylation) and sulfonic acid ($-SO_3H$) (sulfonylation). Increased NO radicals can also attack cysteine and lead to the formation of nitrosothiols (-SNO). Formation of nitrosothiols is known as cysteine nitrosylation that is a reversible process. This process can be reversed by Trx. Trx through its catalytic site thiols react with nitrosothiols of the proteins and convert them to cysteine thiols by the liberation of nitroxyl ion (HNO) and this process is termed as denitrosylation.

In neurons, cysteine oxidative modification of proteins can have several effects on proteins. Previously our laboratory reported that nitrosylation of vesicular monoamine transporter-2 and vesicular glutamate transporter was increased by NO donor S-nitrosoglutathione, and decreased the vesicular dopamine and glutamate uptake in the

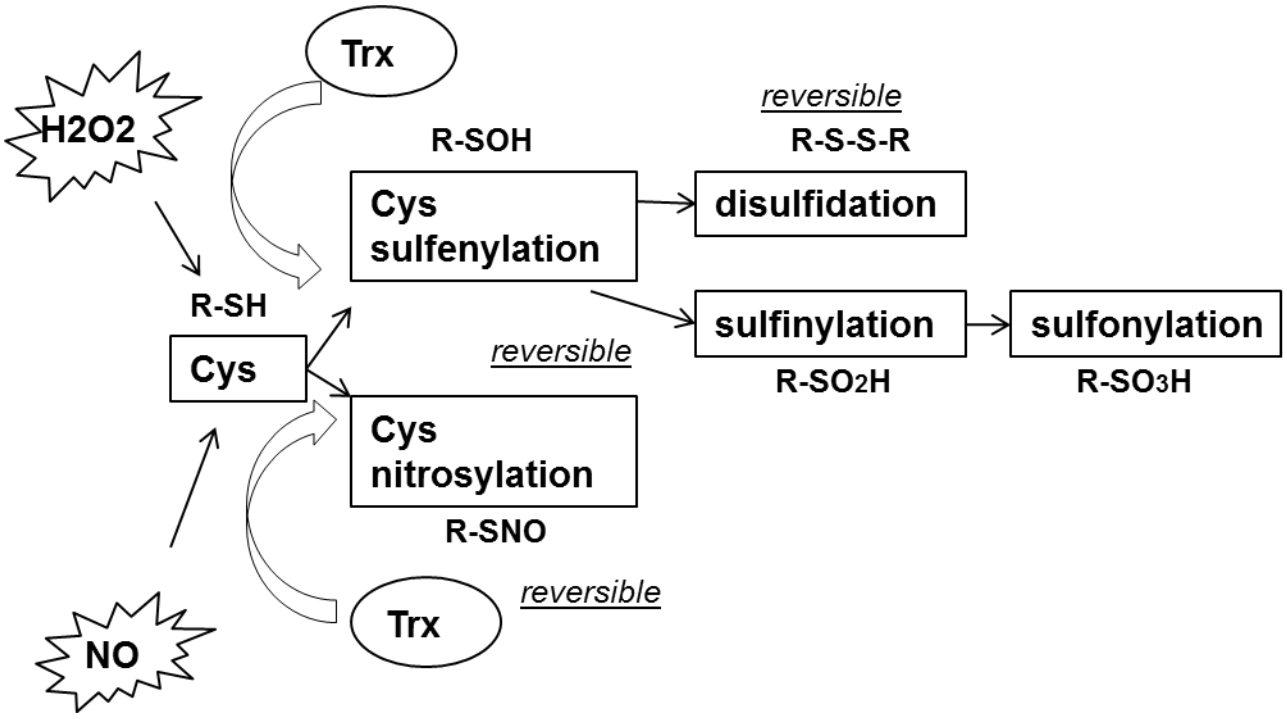


Fig. 4. Functions of Thioredoxin: Attack of H_2O_2 on cysteine thiol leads to formation of sulfenic acid. Sulfenic acid reacts with adjacent cysteine free thiol to generate disulfides. Sulfenic acid upon subsequent oxidation leads to generation of sulfonic and sulfinic acid. Attack of NO on cysteine thiol leads to formation of nitrosothiol.

H_2O_2= hydrogen peroxide, NO= nitric oxide, RSOH= sulfenic acid, RSO_2H= sulfonic acid, RSO_3H= sulfinic acid, RSSR= disulfide, RSNO= nitrosothiol

mouse brain [87], suggesting that the vesicular neurotransmitter uptake may be inhibited by the nitrosylation of these transporters. It has been reported that the nitrosylation of glutamate AMPA receptor enhanced the phosphorylation of glutamate AMPA receptor and conductance[88]. These findings suggest that protein cysteine oxidative modification can regulate neuronal functioning by targeting various proteins.

Trx and peroxiredoxin: Trx can reduce the oxidized form of peroxiredoxin and maintain peroxiredoxin reductase activity. Peroxiredoxin is a family of antioxidant enzymes that can scavenge peroxides including H_2O_2, peroxynitrite, lipid hydroperoxides and others. Once peroxide substrates are reduced by peroxiredoxin, redox-active cysteines in peroxiredoxin active site are oxidized. Trx can reduce these oxidized cysteines back to reduced cysteines and restore peroxiredoxin catalytic activity. Therefore Trx can also inhibit oxidative stress by maintaining peroxiredoxin reductase activity.

Trx and apoptosis signal kinase-1 (ASK-1): Trx can also bind to ASK-1 and inhibit ASK-1 activation. ASK1 belongs to mitogen-activated protein kinase kinase kinase family and can be activated by ROS, inflammatory cytokines, endoplasmic reticulum stress, and calcium influx. Once ASK1 is activated, ASK1 and TNF-α receptor-associated factors form a larger protein complex, which induces ASK1 auto-phosphorylation at residue threonine 845 in mouse and at residue threonine 838 in human. ASK1 phosphorylation then phosphorylates mitogen-activated protein kinase kinase, resulting in phosphorylation of c-Jun-N-terminal-kinase (JNK) and protein 38

(p38). JNK can further phosphorylate c-Jun. The phosphorylated c-Jun dimerizes with c-Fos to form AP-1. AP-1 is a transcription factor that regulates the gene transcription of various cytokines and growth factors and controls cellular differentiation, apoptosis and inflammation. Phosphorylated JNK can also increase the release of cytochrome C from the mitochondria by activating B cell lymphoma (Bcl-2) and Bcl-2 like 1 protein (Bax). This process leads to caspase-3 activation which further leads to cell death. The reduced form of Trx binds to ASK1, subsequently inhibiting apoptosis, oxidative stress, and inflammatory processes.

Trx and gene transcription: Trx can bind to redox-sensitive cysteines of DNA binding domain in a number of transcription factors. It has been studied that there is a direct physical association between DNA binding loop of NF-κB p50 and Trx [89]. To understand the role of Trx, recombinant Trx was incubated with the wild type p50 subunit of NF-κB and cysteine 62 mutant of p50 subunit of NF-κB in Jurkat T cells[89]. DNA binding activity was found to be 3-fold higher for wild type p50 subunit as compared to cysteine 62 mutant p50 subunit, suggesting that Trx interacts with the DNA binding domain of NF-κB p50 via cysteine 62. Trx can directly interact with Ref-1, a nuclear protein that acts as an activator of AP-1. It was shown in Cos-7 cells that co-expression of the wild-type Trx and Ref-1 fusion constructs induced the reporter gene activity more than 10-fold [89] , suggesting that Trx and Ref-1 bind to each other. To further understand the mechanism of this interaction, Trx cysteine 32/35 mutation and Ref-1 fusion constructs were co-expressed. No reporter gene activity was induced, suggesting that Trx binds to Ref-1 through its cysteines present in the catalytic site.

Trx can also directly target the DNA binding domain of GR and thus is responsible for reducing the impaired cellular response to GC under oxidative stress conditions[90]. The HeLa cells were transfected with sense or antisense Trx plasmid, GR expression plasmid, and glucocorticoid-responsive reporter plasmid, and then cultured in the presence of 100 nm dexamethasone and 1mM H_2O_2. H_2O_2 treatment resulted in an approximately 3-fold decrease in GC induction response. The Trx overexpression abolished the repression effect of H_2O_2 while anti-sense Trx resulted in a further decrease in hormone induction by H_2O_2[90], suggesting that there is functional interaction between GR and Trx. Further to analyze the physical interaction between Trx and GR-DNA binding domain (DBD), mammalian two-hybrid assay was performed in CV-1 cells. In these cells, cDNA of GR DBD or Trx was subcloned downstream of GAL4 DBD or the transactivation domain of VP16 protein of herpes simplex virus. These plasmids were co-transfected with a reporter plasmid containing luciferase gene expression under GAL4 binding sites. The co-expression of GAL4-Trx and VP16-GR DBD significantly induced the luciferase expression, suggesting that Trx and GR DBD physically interact with each other [90].

1.5.2. Regulation of thioredoxin

Thioredoxin reductase: Trx activity is maintained by Trx reductase (TrxR) (**Fig. 5)**. TrxR is a homodimeric flavoprotein oxidoreductase. It contains C-terminal selenocysteine in its Gly-Cys-SeCys-Gly and N-terminal -Cys-Xaa-Xaa-Cys active site. In the first step of the catalytic cycle, NADPH reduces the enzyme-bound flavine adenine

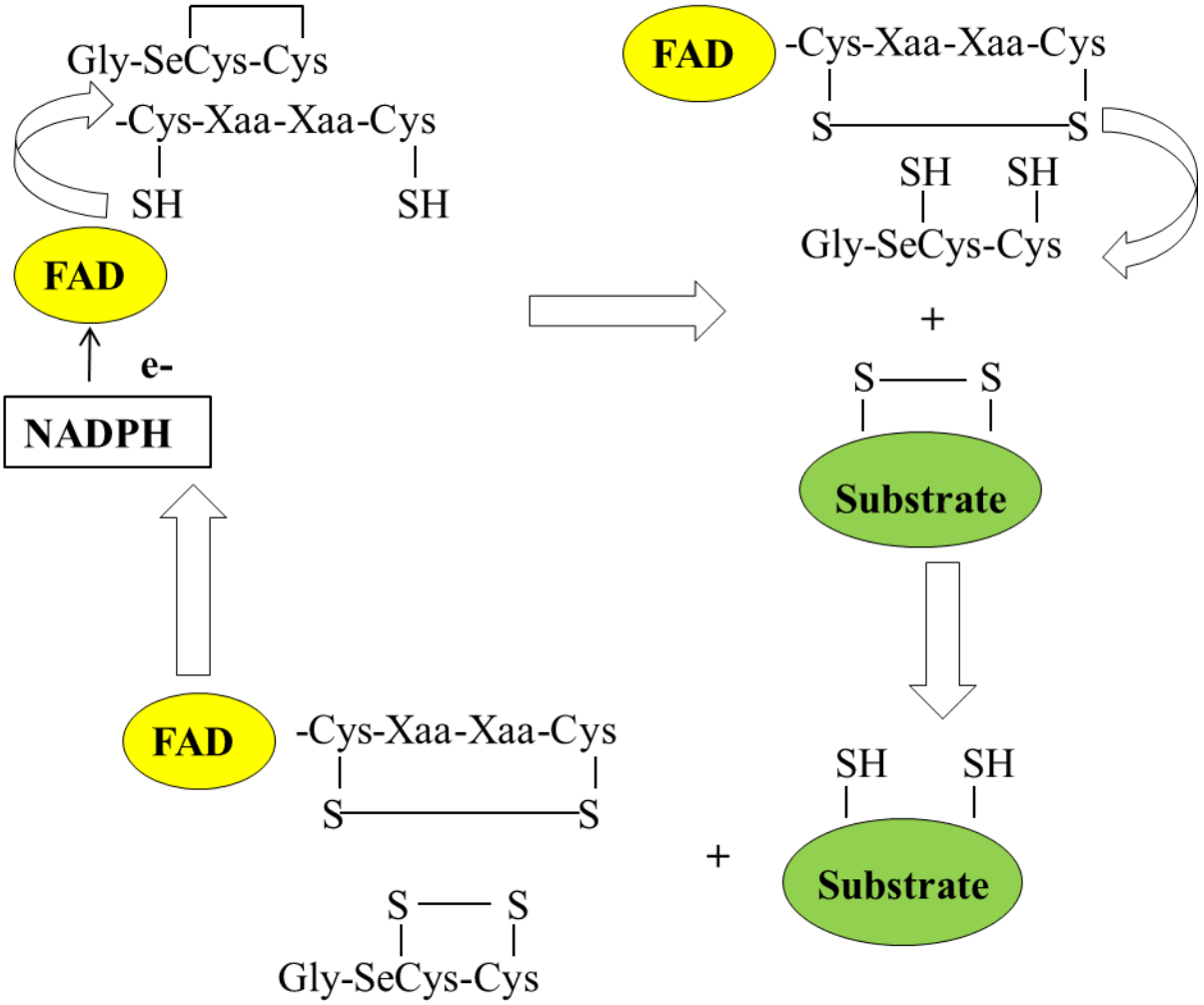


Fig. 5. Regulation of Thioredoxin reductase system: TrxR contains C-terminal selenocysteine in its Gly-Cys-SeCys-Gly and N-terminal -Cys-Xaa-Xaa-Cys active site. First NADPH reduces the enzyme bound FAD. FAD transfers the electrons to the -Cys-Xaa-Xaa-Cys. This site further reduces the C-terminal Gly-Cys-SeCys-Gly site due to thiol disulfide exchange mechanism. Reduced Gly-Cys-SeCys-Gly site then reduces the oxidized substrate protein and it gets oxidized.

Abbreviations: FAD: Flavin adenine dinucleotide, TrxR: Thioredoxin reductase

dinucleotide (FAD). At this time, the C-terminal moiety Gly-Cys-SeCys-Gly is oxidized. Due to the thiol-disulfide exchange mechanism, N-terminal -Cys-Xaa-Xaa-Cys gets oxidized and C-terminal Gly-Cys-SeCys-Gly gets reduced. The reduced Gly-Cys-SeCys-Gly then transfers the electrons to Trx. Similar to Trx, TrxR is also present in two isoforms i.e. TrxR1 in cytoplasm and TrxR2 in mitochondria.

Thioredoxin-interacting protein: Txnip is an approximately 46 kD protein and was originally cloned as a vitamin D_3 target gene in HL-60 cells. Txnip consists of NH2 domain that contains two SH3 binding domains. SH3 domains mediate the scaffold properties of Txnip in various cellular compartments, including cytosol, nucleus, and plasma membrane. The COOH domain contains two PPxY motifs and three SH3 domains. The PPxY domains regulate various functions, such as ubiquitination and degradation of Txnip and facilitation of binding with phosphatase SHP2, which is further known to regulate activation of tyrosine kinase receptors. Txnip performs redox-dependent as well as redox-independent functions. The redox-dependent function includes inhibition of activity of Trx. Txnip binds to the reduced Trx and inhibits the activity of Trx by forming a stable mixed disulfide between the Cys32 residue of Trx and the Cys247 residue of Txnip. Cysteine 63 and 247 make an intramolecular disulfide bond, and cys 247 binds to cys 32 residue of Trx. Redox-independent functions include suppression of cell proliferation and impairments in glucose and lipid metabolism etc.

Post-translational modifications of thioredoxin: The biological functions of Trx can be modulated by various post-translational modifications such as nitrosylation, and

intramolecular disulfide formation, etc. Trx1 in *E.Coli* is susceptible to the irreversible 4-Hydroxy-2-nonenal (HNE) modification. HNE is produced from lipid peroxidation and metabolism. Fang et al., 2006 showed that HNE modified *E.Coli* Trx at Cys 32/35 site[91]. Another study also showed that HNE modified mammalian Trx at Cys73 [92] . Trx is also studied to be S-nitrosylated at Cys 62, 69, or 73 [93–95]. S-nitrosylated Trx1 is known to have anti-apoptotic activity independent of disulfide reductase activity[93].

1.5.3. Chronic stress, glucocorticoids, and Trx system

A few studies indicate that stress and glucocorticoid can regulate Trx system, but results are inconclusive[96,97]. For example, it has been found that acute treatment with 5 mg/kg i.p. dexamethasone for 5 hours increased mRNA and protein levels of mitochondrial Trx in rat amygdala, hippocampus, telencephalon, diencephalon, mesencephalon, and pons[96]. Rats exposed to chronic unpredictable stress for 3 weeks showed a decreased level of Trx protein in the CA1 area of the hippocampus as compared to non-stressed controls[97]. However, our laboratory has found that Trx protein levels were not changed in the hippocampus of mice subjected to chronic unpredictable stress for 28 days when compared to controls[98]. Because we used the whole hippocampus, sub-regional changes of Trx may not be observed. Trx protein levels in CA1, CA3 and dentate gyrus sub-regions of the hippocampus may require to be further investigated. These studies together also suggest that acute stress may compensatorily upregulate Trx to protect against stress-caused oxidative damage, while chronic stress may downregulate Trx that contribute to chronic stress-induced oxidative damage process.

1.5.4. Regulation of Trx in psychiatric disorders

Clinical studies indicate that levels of Trx in various biological fluids are regulated in psychiatric disorders. It has been shown that Trx levels were lower in plasma of the male patients with bipolar mania than controls, indicating the importance of oxidative balance and Trx levels in bipolar disorder [99]. Another clinical study was performed in 80 patients with autism spectrum disorder (ASD), and 100 age and sex-matched normal controls. Serum Trx levels were found to be approximately 3 times higher in ASD patients than in normal controls [100]. This study suggests that elevated levels of serum Trx can be used as a novel independent diagnosis marker for ASD.

Zhang and associates examined the association between serum Trx levels and cognitive deficits in schizophrenia in 45 chronic schizophrenic patients and 66 healthy controls. Cognitive scores were determined by Repeatable Battery for the Assessment of Neuropsychological Status (RBANS). They found that Trx levels were inversely correlated with the attention domain of cognitive deficits in the patient's group, while Trx levels were positively correlated with the attention domain in healthy controls. The differential association of Trx with cognitive functioning in schizophrenia patients and controls suggests that cognitive deficits in schizophrenia might be related to Trx dysregulation in these patients [101]. Recently Bas et al. also measured the relationship between serum Trx levels and cognitive functions in both acute state and remission state in patients with schizophrenia. The authors found that serum Trx levels were highest in patients with acute psychotic episode, lower in patients with remission state and lowest in healthy controls. In this study Trx levels were positively correlated with negative

symptoms, suggesting that Trx may play an important role in the development of negative symptoms. Although the role of Trx is not clear in psychiatric disorders, these results together may suggest that Trx dysregulation may contribute to the pathophysiological development of these disorders.

1.6. Summary and hypothesis

Summary: Studies have consistently shown that chronic stress and excessive GC can impair neuroplasticity, increase ROS production, decrease endogenous antioxidant defense, increase oxidative damage to proteins, lipids and DNA, and increased pro-inflammatory cytokine levels in cultured neuronal cells and animals. These findings suggest that chronic stress and excessive GC may cause oxidative stress and neuroinflammation, resulting in impairment of neuroplasticity. Chronic stress is a major risk factor for MDD and other psychiatric disorders. Oxidative stress and inflammation are also increased in MDD patients. Antidepressants are medications that can treat MDD in clinical settings. Previous studies have shown that chronic treatment with antidepressants can inhibit oxidative stress and inflammation, suggesting that oxidative stress and inflammation may contribute to the pathophysiological development of MDD and can be targeted by the pharmacological treatment for MDD. Trx antioxidant system is composed of Trx, TrxR, and Txnip. Trx can reverse cysteine oxidative modification such as sulfenylation and nitrosylation, scavenge H_2O_2 and other peroxides, and inhibit ROS and inflammatory cytokine regulated ASK1 signaling. TrxR maintains Trx in an active state, while Txnip is an endogenous inhibitor. Since Trx antioxidant system plays an important role in defense against oxidative stress and inflammation, deficiency of this

system may contribute to chronic stress and excessive GC-induced oxidative stress and inflammation.

Hypothesis: Prolonged CORT treatment downregulates Trx and TrxR, but upregulates Txnip, resulting in oxidative stress and neuroinflammation.

1.7. Overall objectives of the project

The objectives of this project are:

1. To determine the effect of chronic CORT treatment on Trx, TrxR, and Txnip protein levels in cultured neuronal cells
2. To determine the effect of chronic CORT treatment on protein cysteine oxidative modification in cultured neuronal cells
3. To determine the effect of chronic antidepressant treatment on Trx, TrxR, and Txnip protein levels, and cysteine oxidative modification in cultured neuronal cells
4. To determine the effect of chronic CORT treatment on Trx, TrxR, and Txnip protein levels and inflammatory signaling in cultured microglia

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell culture

2.1.1. Culture of HT22 mouse hippocampal cell line and N9 mouse microglia cell line

The Salk Institute (La Jolla, CA, USA) generously provided the HT22 cells, while N9 cells were generously provided by Professor Maria-Grazia Martinoli (Universite du Quebec a Trois-Rivieres, Quebec, Canada). These cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Life Technologies Inc, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and kept in the incubator under 5% carbon dioxide (CO_2) at 37°C.

2.1.2. Primary culture of mouse cerebral cortical neurons and microglia

Primary culture of cerebral cortical neurons was performed as described previously[102]. The embryonic fetuses were removed from the uterus of the mouse with 17-18 days gestation. The cerebral cortex was dissected by Dr. Michael Jackson's lab and provided to us. The meninges and blood vessels were removed. The cortex was minced finely and tissue was digested with 0.25% trypsin for 15 min at 37°C followed by DNAse (0.7 mg/ml) digestion for the next 15 min at 37°C. Trypsinization procedure was stopped using 10% of fetal bovine serum.

After precipitation, the supernatant was discarded, and the cells were dissociated mechanically by pipetting and resuspended in 1 ml of neurobasal medium (Life Technologies Inc.) containing 1x Glutamax (Life Technologies Inc.), 1x GS21 supplement (Sigma-Aldrich, Canada) and 1% penicillin/streptomycin. The cells were

seeded in poly-D-lysine coated (50 µg/ml) 6-well cell culture plates at a density of $3x10^5$ cells/ml resulting in 99.98% pure neuron cultures as previously done by our laboratory [102]. Cells were maintained at 37°C under 5% CO_2 for 10 days before CORT treatment. The half medium was changed twice per week.

Primary culture of mouse microglia was performed as described previously[103]. Cerebral cortex from embryonic fetuses was isolated as described above. The cortex was minced finely and transferred to a 15 ml tube to allow the tissue to settle down. Once the tissue was settled down, media was removed. Then 500 µl of 0.25% trypsin was added to the tissue and incubated for 3 min at 37°C. 2 ml of DMEM media containing 10% FBS and 1% streptomycin/penicillin was also added to terminate the digestion process. The supernatant was removed and tissue was resuspended with DMEM media containing 10% FBS and 1% streptomycin/penicillin. Media was changed at day 2, 4 and 7. To collect microglia, flasks were vigorously tapped on the bench top and collected in a 15 ml tube. Cells were plated at a density of $3x10^5$ cells/ml on a 6-well plate resulting in 99% pure microglial culture as described previously[103]. The cells were maintained at 37°C under 5% CO_2.

2.2. Protein isolation

Cultured cells were washed twice with phosphate buffer saline (PBS), then scraped with 500 µl of PBS, and collected in an Eppendorf tube. These cells were centrifuged at 1000 *g* for 5 min at 4°C. The supernatant was removed, and 50 µl of ice-cold lysis buffer containing 20 mM HEPES (pH 7.5), 20% glycerol, 0.5 mM EDTA, 250 mM NaCl,

0.1mM ethylene glycol tetraacetic acid (EGTA), 30 mM $MgCl_2$, 1% nonidet P40 and 1× protease inhibitor cocktail (Thermo Scientific, Marietta, OH, USA) was added to the cell pellet. The cells were vortexed and kept on ice for 45 min and centrifuged at 10,000 *g* for 10 min at 4°C. The cell pellet was removed and supernatants were collected and used as protein extract. Protein concentration was determined by Bradford protein assay [104].

2.3. Immunoblotting analysis

Sample buffer containing 100mM Tris-HCl (pH 6.8), 200 mM dithiothreitol (DTT), 0.2% bromophenol blue, 4% sodium dodecyl sulfate (SDS), and 20% glycerol was added to the proteins, and then were loaded to 12% SDS polyacrylamide gel and subjected to electrophoresis at 120 V for 1 h. Then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) for 2 hours at 220 mA on ice. Membranes were blocked at room temperature for 1h with 5% milk in Tris-buffered saline (TBS) containing 10mM Tris-HCl (pH 7.4) and 0.1% Tween-20. The membranes were then incubated overnight at 4°C with primary antibodies including: rabbit monoclonal Txnip (1:2000 dilution, Abcam Inc., Toronto, ON, Canada), rabbit monoclonal Trx1 (1:7000 dilution, Cell Signaling Technology, Danvers, MA, USA), TrxR (1:1000 dilution, Santa Cruz Biotechnology Inc., CA), NLRP3 (1:2000 dilution, Adipogen, CA, USA), caspase-1 (1:2000 dilution, Adipogen, CA, USA), IL-1β (1:2000 dilution, R&D systems, MN, USA); followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The chemiluminescence system (Bio-Rad, Dreieich, Germany) was used to analyze the immunoreactive bands and imaged using the ChemiDoc MP System (Bio-Rad). The Image Lab software was used to

quantify the band signal intensity (Bio-Rad).

2.4. ROS measurement

ROS was measured using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described previously[105]. H_2DCFDA is chemically reduced and acetylated form of fluorescein. Upon cleavage the acetate group by esterase present in cells and oxidation, this is converted to fluorescent 2',7'-dichlorofluorescein (DCF). Cells were seeded at a seeding density of 3×10^6 cells/well, and then treated with CORT for 5 days. After 5 days the cells were washed with PBS. 100 µl of 25 µM H_2DCFDA (Molecular Probes, Canada) was added to the cells and incubated for 30 min in dark at 37 °C. After 30 min, cells were washed with 100 µl PBS. H_2O_2 at 150 µM was used as a positive control. Fluorescence was measured using excitation and emission wavelengths at 485/528 nm. Blank readings were subtracted from all measurements.

2.5. NO measurement

Nitrite is a stable breakdown product of NO. NO was analyzed by measuring nitrite. Nitrite was measured using Griess reagent as previously described[106]. Griess reagent consists of sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED). Sulfanilamide reacts with nitrite to make an intermediate compound that further reacts with NED to generate colored detectable azo compound. Sodium nitrite ($NaNO_2$) standard was prepared in the concentration range of 0-250 µM by performing serial dilution. Griess reagent was freshly prepared by dissolving 0.05 g sulfanilamide (Sigma Aldrich, Canada) and 0.005 g NED (Sigma Aldrich, Canada) in 4.885ml ddH_2O and 115

µl of 85% phosphoric acid (Sigma Aldrich, Canada) and then vortexed. In a 96 well plate, 50 µl of $NaNO_2$ standard was added in each well in triplicate. 50 µl of the sample (cell culture supernatant) was also added in triplicate. 50 µl of Griess reagent was added to wells containing standards and samples. Absorbance was measured at 540 nm after 10-20 min of incubation at room temperature using SkanIt software 4.1 (Thermo Scientific, Canada). Cell extracts were also lysed using lysis buffer. The data was normalized by dividing absorbance/ protein (mg/ml).

2.6. Dimedone conjugation assay for detection of cysteine sulfenylated proteins

The detection of cysteine sulfenylated proteins was done by dimedone conjugation method as previously described by Seo and colleagues with minor modifications[107]. The principle behind this assay is that dimedone forms the thioester bond with sulfenic acid that can be further analyzed by western blotting using dimedone conjugated anti-sulfenic acid antibody. First, the HT22 cells were washed with PBS and digested with 0.25% trypsin. The trypsinization process was terminated using DMEM containing 5% dimedone and 10% fetal bovine serum. The cells were centrifuged at 1000 *g* and the supernatant was discarded. The cell pellets were lysed in a buffer containing 2 mM dimedone, 3 mM citric acid, 0.1% Triton-X, 12 mM sodium phosphate dibasic, 10 mM iodoacetamide, 10 mM *N*-ethylmaleimide, 10 mM EDTA, 200 units/ml catalase, and 0.01% SDS, and incubated on ice for 30 min. Then the lysates were centrifuged at 10,000g for 10 min followed by the collection of the supernatants. Bradford protein assay[104] was used to quantify the protein concentrations. 15 µg of protein was loaded to 12% SDS/PAGE and the sulfenylated proteins were analyzed using western blotting with

1:5000 dilution of anti-cysteine sulfenic acid antibody (Millipore Canada Ltd, Etobicoke, ON, Canada).

2.7. Biotin-switch assay for detection of cysteine nitrosylated proteins

Cysteine nitrosylated proteins were detected by the biotin-switch assay as previously described[87]. The blocking buffer was prepared by adding 2.5% SDS and 20 mM methanethiosulfonate (MMTS) in HEN buffer containing 250 mM HEPES pH 7.7, 1 mM EDTA and 0.1 mM neocuproine). 200 µl of blocking buffer was added to 50 µg of protein and incubated for 40 min at 50°C. Then 1 ml of pre-chilled acetone was added to the reaction mixture at -20 °C for 35 min. The samples were centrifuged at 15,000 *g* at 4°C for 15 min and supernatants were removed. Another buffer was prepared by adding 1% SDS to HEN buffer. 5 µl of this buffer was added to the protein pellets and vortexed in order to resuspend the proteins. Then the nitrosylated cysteine thiols were reduced back to free thiols using ascorbate. The reduced thiols were further labeled with *N*-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP). To the resuspended pellets, 250 µl of 50 mM ascorbate and 15 µl of 4 mM biotin-HPDP were added and incubated at room temperature for 1 h. Nitrosylated proteins were analyzed using western blotting with 1:3000 polyclonal anti-biotin antibody (Sigma, St. Louis, MO, USA).

2.8. Knocking out of Txnip by CRISPR/Cas9

HT22 Txnip knockout cells were generated by Dr. Eftekharpour's laboratory, while N9 Txnip knockout cells were generated in our laboratory. To knock out HT22 cells,

CRISPR-Cas9 technology by lentiviral transfection approach was used. Txnip scrambled guide RNA (sgRNA), and CRISPR/Cas9 All-in-One lentivector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro were purchased from ABM Inc. (Richmond, BC, Canada). The HEK293T cells were co-transfected with packaging plasmids (GAG, VSVG, REV and TAT) and Cas9-sgRNA constructs, and the viral particles were generated as described previously[108]. The conditioned media containing lentiviral particles was harvested. HT22 cells were transfected with virus particles using polybrene as transfection agent. 4 µg/ml Puromycin was used in cell culture media to select and generate stable Txnip knockout HT22 cells. The single colonies from the scramble and Txnip knockout cells were picked and grown for further experiments. We also used CRISPR-Cas9 technology to knock out Txnip in N9 mouse microglia. Txnip scrambled guide RNA (sgRNA), and CRISPR/Cas9 All-in-One lentivector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro were purchased from ABM Inc. (Richmond, BC, Canada). The HEK293 T cells were co-transfected with packaging plasmids (GAG, VSVG, REV, and TAT) and Cas9-sgRNA constructs. The viral particles were generated as described previously [108]. The conditioned media containing viral particles was harvested and stored at -80° C. N9 cells were transfected with virus particles. Puromycin was added in cell culture media to select and generate stable N9 Txnip knockout cells. The single colonies from scramble and Txnip knock out cells were picked and cultured for further experiments.

2.9. Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously [109] . 100 µg of proteins from N9 cells, mouse hippocampus or frontal cortex were incubated with mouse

monoclonal anti-NLRP3 (dilution 1:500, Adipogen, CA, USA) overnight at 4°C. Then 7 µl of protein A/G beads (Santa Cruz Biotechnology, USA) were added to protein-antibody complex and incubated for 2 h at 4°C. Protein-bead mixture was then centrifuged at 1000 g for 30 seconds and supernatants were removed. The beads were washed three times with cold lysis buffer containing 50 mM Tris-HCl (pH=7.4), 150 mM NaCl, 1mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Thermo Scientific, Marietta, OH, USA). After the final wash, 15 µl of sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue and 10% glycerol was added to the beads. The samples were heated at 95 °C for 5 min and were subjected to SDS/PAGE and transferred to PVDF membranes as described above. The membrane was incubated with primary antibody poly rabbit anti-Txnip (1:2000 dilution, Abcam Inc., Toronto, ON, Canada) overnight at 4°C followed by incubation with secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. The chemiluminescence system (Bio-Rad, Dreieich, Germany) was used to analyze the immunoreactive bands and imaged using the ChemiDoc MP System (Bio-Rad). The Image Lab software was used to quantify the band signal intensity (Bio-Rad).

2.10. Measurement of caspase-1 activity

The caspase-1 activity was measured as described previously [110]. 100 µg of N9 cell extracts were added to 50 µl of 2X stock reaction buffer containing 50 mM Hepes (pH=7.4), 100 mM NaCl, 10 mM DTT, 1 mM EDTA and 10% v/v glycerol. Then 50 µM of fluorogenic substrate Z-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin **(**Z-YVAD-AFC) (caspase-1 substrate VI, Cayman Chemical, CA) was added to this reaction

mixture and incubated for 2 h at room temperature. After incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured by measuring the fluorescence (excitation 400 nm, emission 505 nm).

2.11. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay kit was purchased from R&D systems, CA and performed according to the manufacturer's instructions. Briefly, first, the 96-well plate was coated with the capture antibody at 4 µg/ml concentration and incubated overnight at 4°C. Then the antibody was removed and the plate was washed 3 times with washing buffer containing 0.05% Tween-20 in PBS at pH=7.4. The plate wells were blocked by using blocking buffer containing 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The plate was washed 3 times. 100 µl of sample or caspase-1 standard was added and incubated for 2 h at room temperature. After washing, 100 µl of detection antibody at a final concentration of 500 ng/ml was used and incubated at room temperature for 2 h. The plate was washed thrice and100 µl of Streptavidin-HRP was added. The plate was incubated for 20 min at room temperature in dark. The plate was washed and 100 µl of substrate solution containing 1:1 mixture of tetramethylbenzidine and H_2O_2 was added to the plate. The plate was incubated in dark for 20 min. Then 50 µl of stop solution containing 2 N H_2SO_4 was added. The optical density was measured by subtracting the readings at 540 nm from readings at 450 nm.

2.12. Statistical analysis

We used IBM SPSS 24.0 software (IBM, Armonk, New York, USA) to perform statistical analysis. We expressed all the results as the mean ± standard error of the mean (SEM). Significant differences among means were analyzed by one-way analysis of variance (ANOVA) with Tukey post hoc comparisons. For the statistical analysis of the two groups Student's t-tests was used. A p value of less than 0.05 was regarded as statistically significant.

CHAPTER 3: RESULTS

3.1. To determine the effect of CORT on Trx system in neuronal cells

3.1.1. To measure the effect of CORT on Trx, TrxR, and Txnip protein levels in HT22 cells and primary cultured cerebral cortical cells

First, we analyzed the effect of long term CORT treatment on the protein levels of Trx, TrxR, and Txnip in HT22 mouse hippocampal cells. We used chronic CORT treatment to mimic the model of chronic stress. Previously studied animal models of chronic stress has shown elevated circulating serum levels of CORT [111,112]. Chronic treatment with CORT induced the depression-like phenotype in animal models[113,114] . CORT animal model and depressed patients also showed similarity such as reduced hippocampal volume[115,116]. We have used HT22 cells in our cell culture model because they contain GR and not the MR that are responsible for chronic stress-induced pathophysiological state. HT22 cells were treated with CORT for 5 days at 0.5, 1 and 2 μM concentrations. Immunoblotting analysis was used to measure the protein levels of Trx, TrxR, and Txnip. We didn't find any effect of CORT on protein levels of Trx and TrxR but found increased levels of Txnip protein ($p<0.05$, N=6). CORT treatment at 0.5, 1 and 2 μM for 5 days in HT22 cells increased Txnip protein levels by 511± 69%, 657± 76% and 526± 129% respectively when compared to control (**Fig. 6**).

The effect of chronic CORT treatment on protein levels of Trx, TrxR, and Txnip was also verified in primary cultured mouse cerebral cortical neuronal cells. CORT treatment was done at 0.5, 1 and 2 μM concentrations for 5 days in primary cerebral cortical neuronal cells. The protein levels of Trx, TrxR, and Txnip were measured by western blotting. We

didn't find any effect of CORT on the protein levels of Trx and TrxR protein levels (**Fig. 7**). We found that CORT significantly increased the protein levels of Txnip at 1 and 2 μM concentrations ($p<0.05$, N=4) (**Fig.7**).

3.1.2. To determine the role of GR in the regulation of Txnip by CORT in HT22 cells and primary cortical neurons

To determine if GR mediates CORT-increased Txnip expression, we measured the effect of GR inhibitor RU-486 on CORT-increased protein levels of Txnip in HT22 cells and primary cultured cerebral cortical cells. HT22 cells and primary cultured cerebral cortical cells were treated with vehicle, CORT at 1 μM, RU-486 at 1 μM, and CORT plus RU-486 for 5 days. As shown in **Fig. 8**, RU-486 itself didn't show any effect on protein levels of Txnip but significantly inhibited the increased Txnip protein levels by CORT treatment ($p<0.05$, N=6), suggesting that CORT increased Txnip protein levels is mediated by GR.

3.2. To determine if CORT induces protein cysteine oxidative modification

3.2.1. To determine if CORT induces cysteine sulfenylation and nitrosylation

Txnip can inhibit Trx activity, subsequently enhancing protein cysteine sulfenylation and nitrosylation. CORT-increased Txnip may further promote protein cysteine sulfenylation and nitrosylation. Therefore, we measured the effect of CORT on protein cysteine sulfenylation and nitrosylation in HT22 cells.

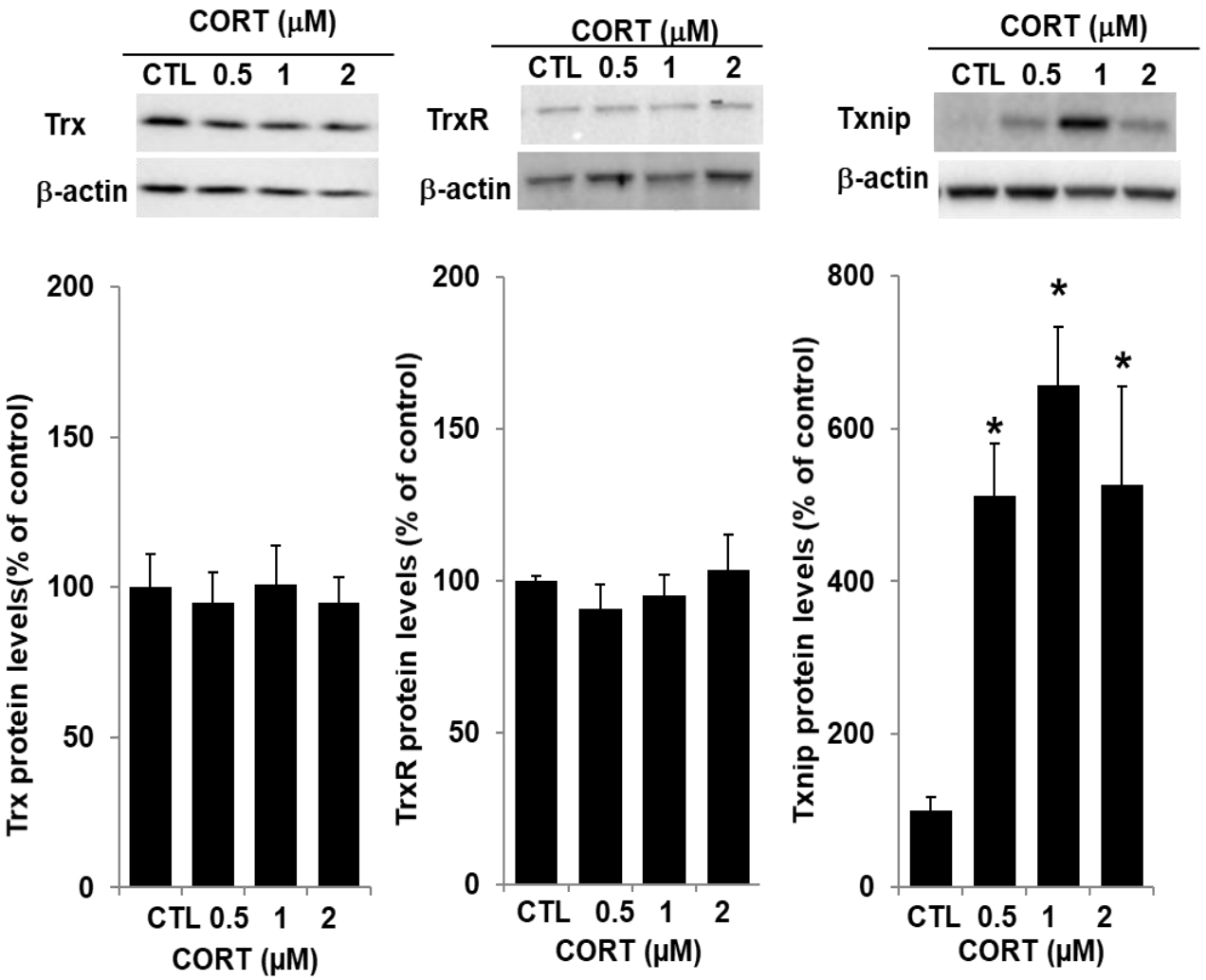


Fig. 6: The effect of corticosterone on Trx, TrxR, and Txnip protein levels in HT22 cells. HT22 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Protein levels of Trx, TrxR, and Txnip were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

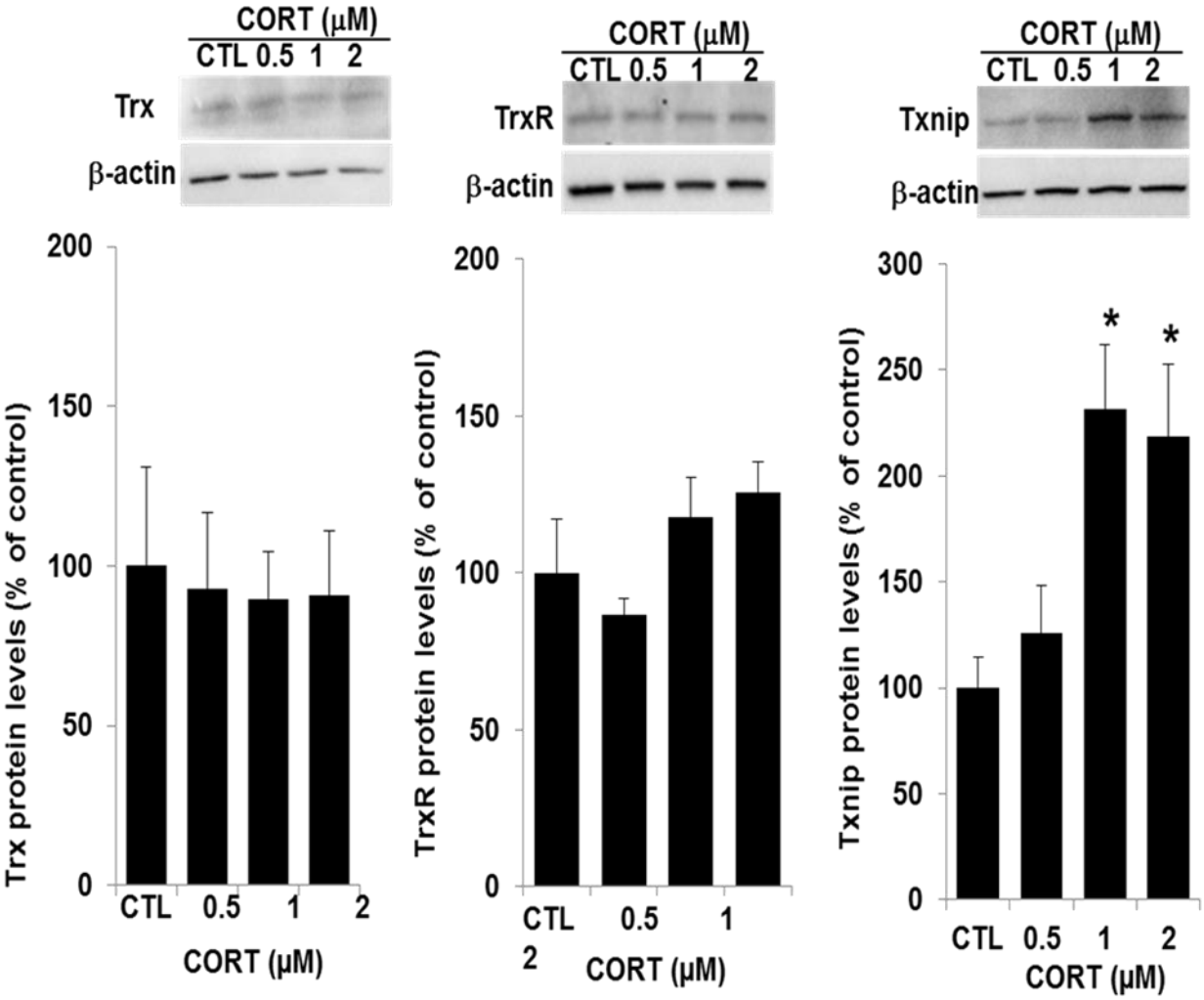


Fig. 7: The effect of corticosterone on Trx, TrxR, and Txnip protein levels in primary cerebral cortical neuronal cells. Primary cerebral cortical neuronal cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Protein levels of Trx (A), TrxR (B), and Txnip (C) were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =4). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

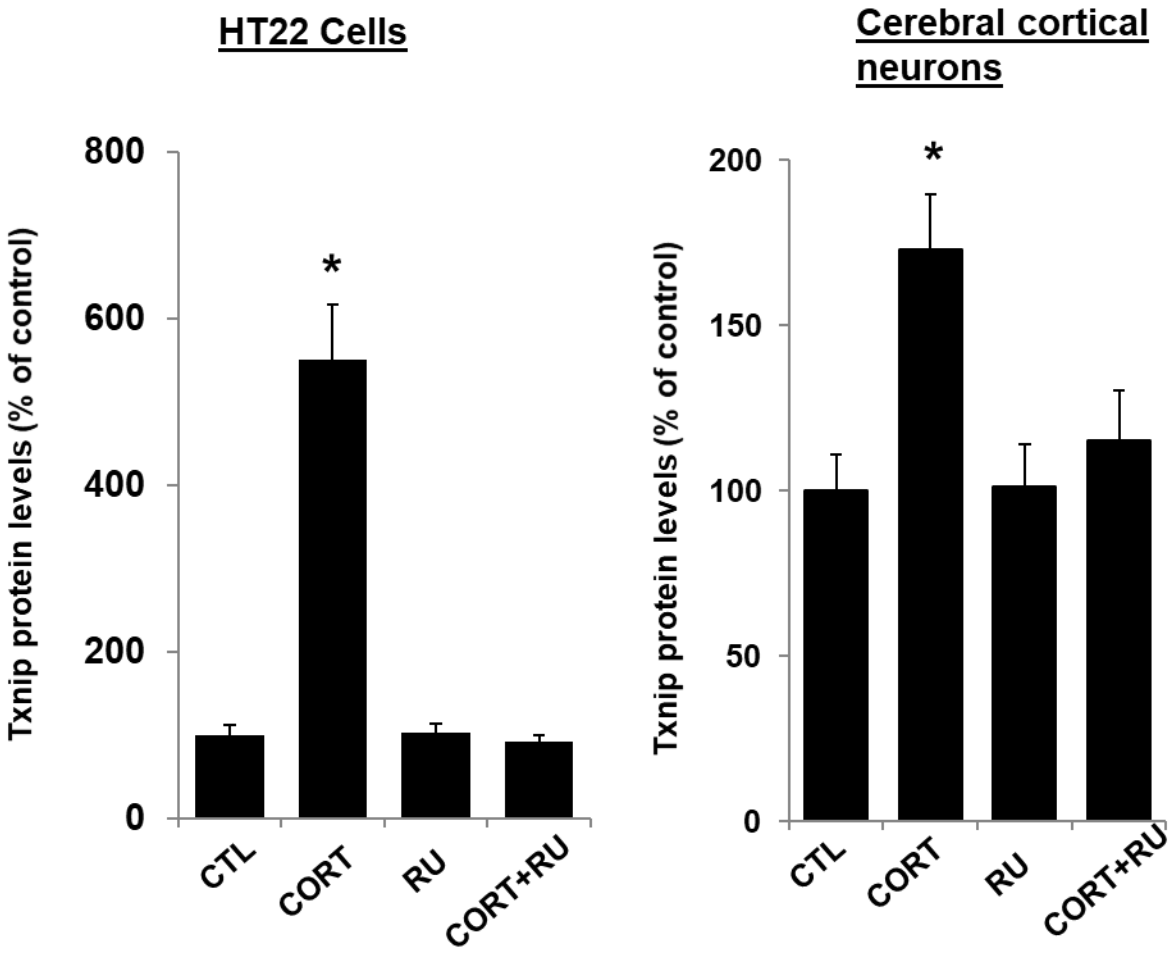


Fig. 8: The effect of GR antagonist RU-486 on Txnip protein levels in HT22 and primary cerebral cortical neuronal cells. HT22 cells (A) and cerebral cortical neurons (B) were treated with CORT and RU-486 for 5 days and then Txnip protein levels were measured. Protein levels of Txnip were measured with Txnip antibody using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

H_2O_2 and NO can attack cysteine and induce cysteine sulfenylation and cysteine nitrosylation respectively. Therefore H_2O_2 was used as a positive control for sulfenylation, while NO donor S-*nitrosoglutathione* (GSNO) was used as a positive control for nitrosylation. Cells were treated with H_2O_2 at concentrations of 75, 150 and 300 μM or treated with GSNO at concentrations of 40, 80 and 160 μM for 30 min. Cysteine sulfenylation was measured by dimedone conjugation assay followed by western blotting. Biotin-switch method was used to measure the protein nitrosylation. As shown in **Fig. 9**, treatment with H_2O_2 at 75, 150 and 300 μM dose-dependently increased sulfenylated protein levels, and treatment with GSNO at 40, 80 and 160 μM also dose-dependently increased nitrosylated protein levels. Next, we measured the effect of chronic CORT treatment on protein cysteine sulfenylation and nitrosylation. CORT treatment was done in HT22 cells at concentrations of 0.5, 1 and 2 μM for 5 days. Although CORT at 0.5 μM has no effect on sulfenylated protein levels, CORT at 1 and 2 μM significantly increased sulfenylated protein levels ($p<0.05$, N=8) (**Fig. 10 A**).
As shown in **Fig. 10 B,** chronic treatment with CORT at 1 and 2 μM also significantly increased nitrosylated protein levels ($p<0.05$, N=6).

3.2.2. To determine if Txnip knocking out reverses CORT-induced cysteine sulfenylation and nitrosylation

To determine if Txnip mediates CORT-increased protein cysteine sulfenylation and nitrosylation, we analyzed the effect of Txnip knockout on CORT-increased protein cysteine sulfenylation and nitrosylation in HT22 cells. Txnip gene was knocked out by the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology.

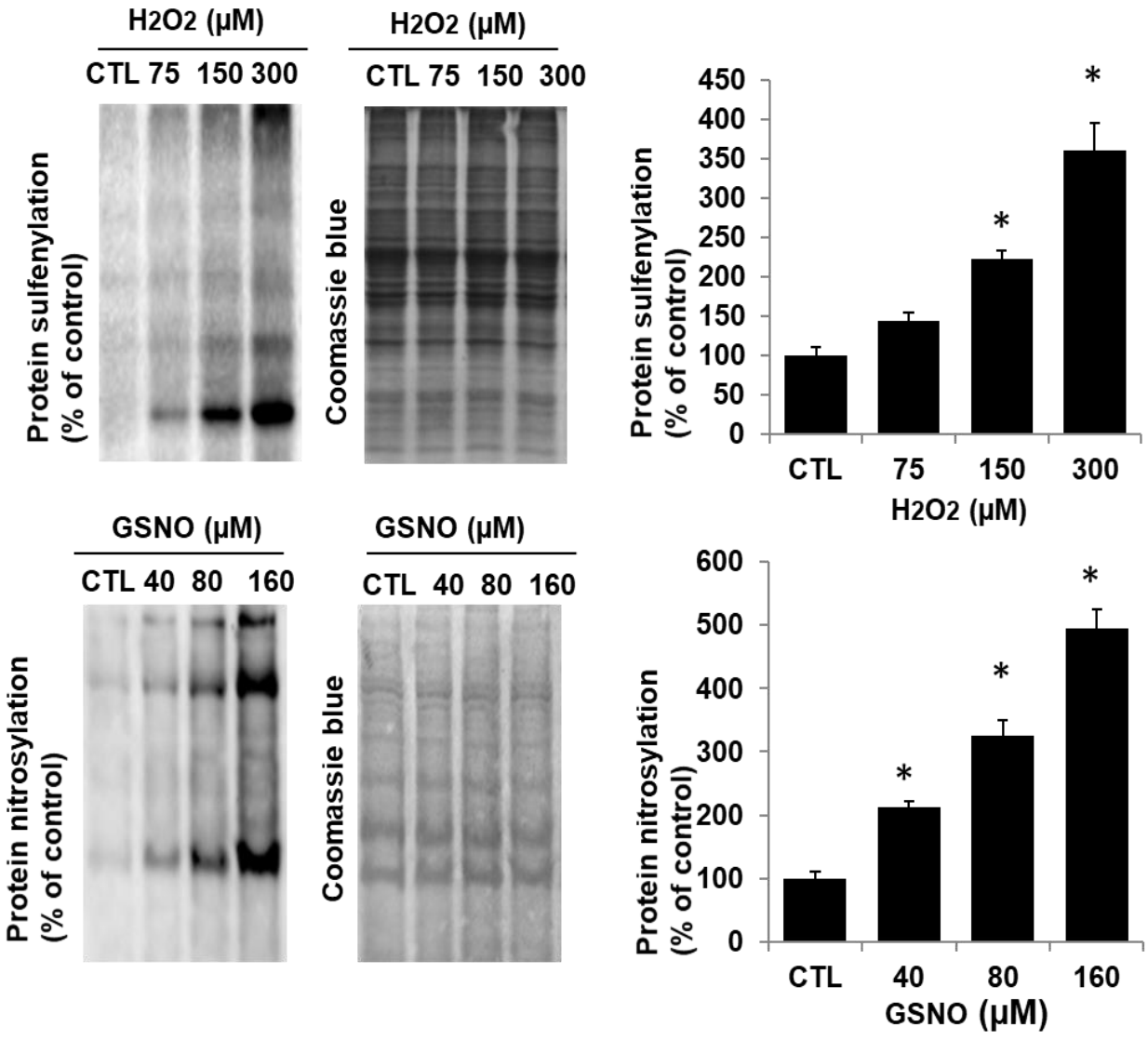


Fig. 9. The effect of H_2O_2 on protein sulfenylation and GSNO on protein nitrosylation in HT22 cells. Cells were treated with vehicle (CTL) or H2O2 (A) to measure protein sulfenylation and GSNO (B) for measurement of protein nitrosylation for 30 min. Sulfenylated protein was measured by dimedone conjugation followed by immunoblotting analysis. Nitrosylated protein was measured by biotin switch followed by immunoblotting analysis Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 3). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

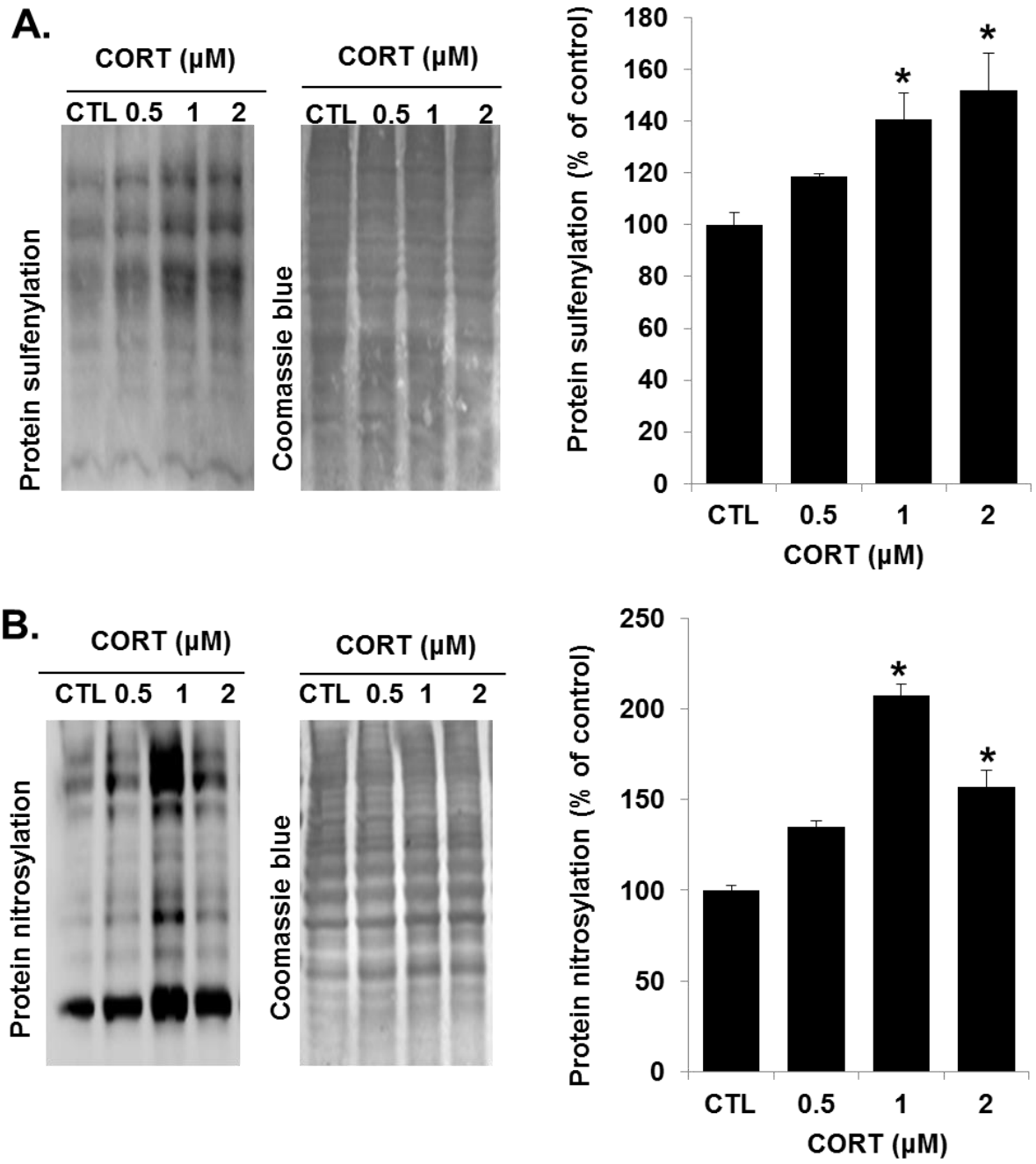


Fig. 10. The effect of corticosterone on protein sulfenylation and nitrosylation in HT22 cells. Cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Sulfenylated protein was measured by dimedone conjugation followed by immunoblotting analysis (A). Nitrosylated protein was measured using biotin-switch method followed by immunoblotting analysis (B). Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 6-8). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

As shown in **Fig. 11 A**, the Txnip single guide RNA (sgRNA) is a short synthetic RNA composed of a scaffold sequence that binds to RNA-guided DNA endonuclease Cas9 and a 20 nucleotide spacer sequence that is complementary to the specific Txnip gene. The Cas9 and the sgRNA can form a ribonucleoprotein complex, and then the Txnip DNA sequence is cleaved by Cas9. The plasmid containing CRISPR/Cas9/Txnip sgRNA or CRISPR/Cas9/scrambled sgRNA was packaged by Lentiviral Core Service at the University of Manitoba. HT22 cells were transfected with lentivirus-packaged Txnip sgRNA/CRISPR/Cas9 plasmid. Scrambled sgRNA were used as control. Then the protein levels of Txnip were measured using western blotting. As shown in **Fig. 11 B**, Txnip protein levels were significantly knocked out in cells transfected with Txnip sgRNA compared to the cells transfected with scramble sequence. This result suggests that Txnip sgRNA totally blocked Txnip gene expression ($p<0.05$, N=5).

Next, the protein sulfenylation and nitrosylation were measured in CORT treated Txnip knockout HT22 cells. Both scrambled and Txnip knock out cells were treated with vehicle or CORT at 1 μM for 5 days. We found that in the cells transfected with scrambled sequence sulfenylated protein levels were increased by CORT treatment but no effect was shown by CORT Treatment in Txnip Knock out cells upon CORT treatment (**Fig. 12 A**) ($p<0.05$, N=4). As shown in **Fig. 12 B**, treatment with 1 μM CORT significantly increased nitrosylated protein levels in cells transfected with scrambled sgRNAs, but has no effect on nitrosylated protein levels, suggesting that Txnip knockout inhibited CORT-increased protein cysteine sulfenylation and nitrosylation in HT22 cells.

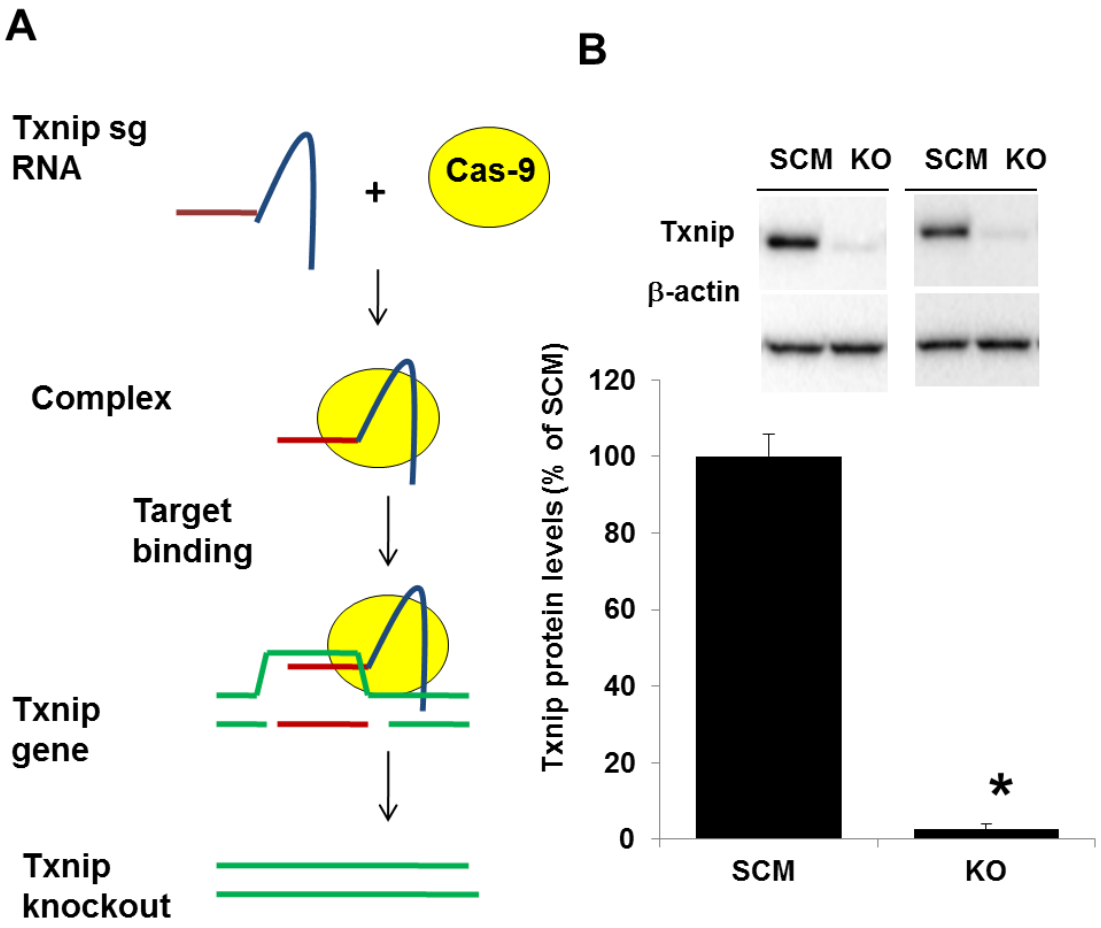


Fig. 11. Txnip gene knockout in HT22 cells using CRISPR-Cas9 technology. Lenti-vectors containing Txnip sgRNAs/CRISPR/Cas9 (KO) or scrambled sgRNAs/CRISPR/Cas9 (SCM) were transfected into HT22 cells. Txnip protein levels were measured by immunoblotting analysis. Data are displayed as mean ± SEM (N = 5). * indicates $p < 0.05$ when compared to SCM group determined by T test.

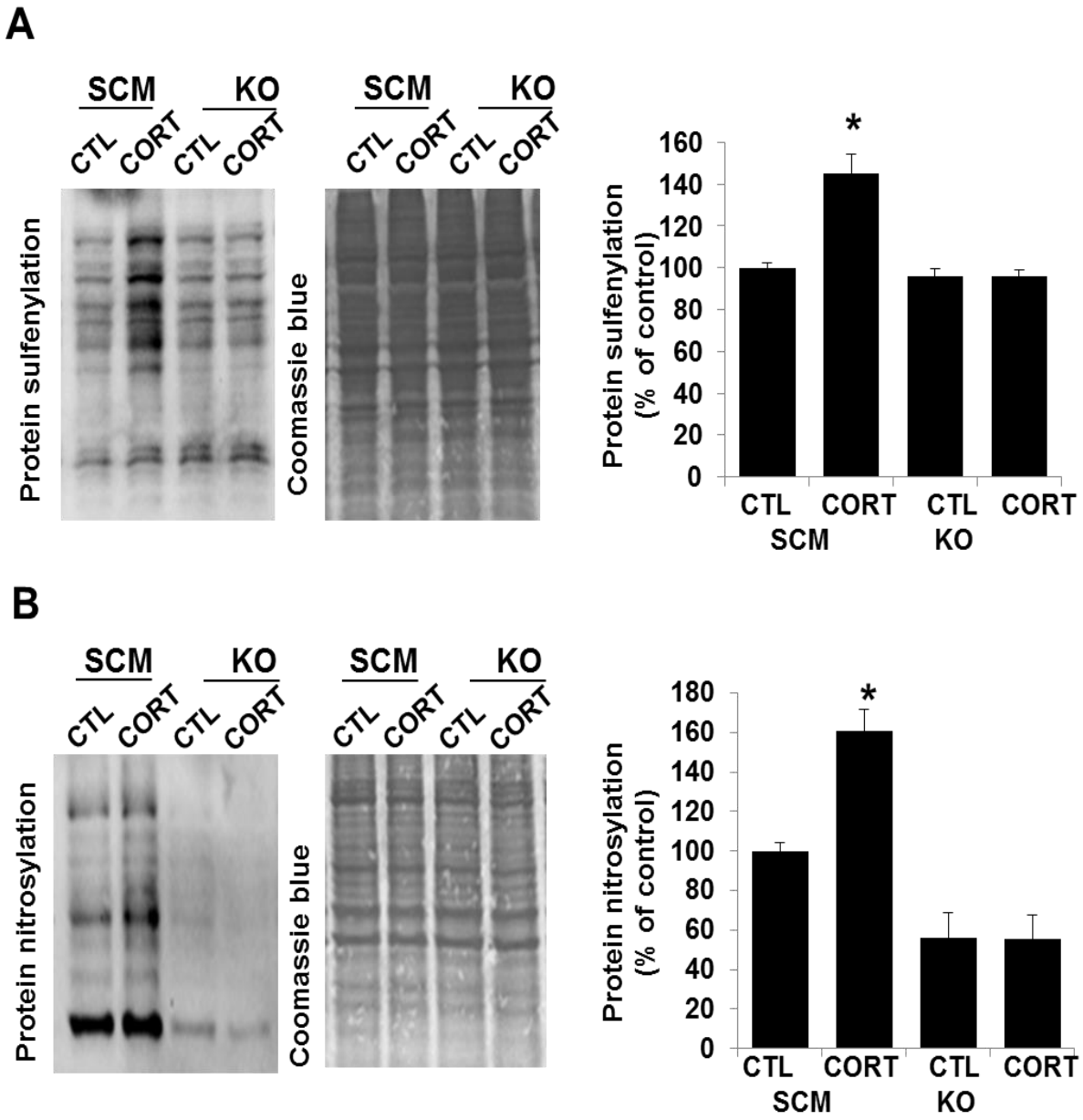


Fig. 12. Effect of Txnip sgRNA on corticosterone-increased protein sulfenylation and nitrosylation. HT22 cells transfected with Txnip sgRNA (KO) or scrambled sgRNA (SCM) were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Sulfenylated protein was measured by dimedone conjugation method (A) and nitrosylated protein was measured by biotin-switch method (B). Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 4). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

3.2.3. To measure the effect of chronic CORT treatment on ROS and NO levels

H_2O_2 and NO radical can attack cysteine thiol groups and cause protein cysteine sulfenylation and nitrosylation respectively. Chronic CORT treatment may also promote protein cysteine sulfenylation and nitrosylation by increasing ROS and NO levels. Therefore we also measured the effect of chronic CORT treatment on ROS and NO levels in HT22 cells. HT22 cells were treated with CORT at concentrations of 0.5, 1 and 2 μM for 5 days. ROS was measured by H_2DCFDA. As shown in **Fig. 13 A**, although chronic treatment with CORT at 0.5 μM has no effect on ROS levels, CORT at 1 and 2 μM significantly increased ROS levels ($p<0.05$, N=4). Chronic CORT at 1 and 2 μM significantly increased ROS levels when compared to control. Chronic treatment with CORT at 0.5, 1 and 2 μM also significantly increased NO levels ($p<0.05$, N=4) (**Fig. 13B**).

3.3. To determine the effect of antidepressants on the Trx system in HT22 cells

3.3.1. To measure the effect of antidepressants on Trx, TrxR, and Txnip in HT22 cells

Chronic stress is a major risk factor for depression. Since chronic treatment with stress hormone CORT upregulates Txnip, we also measured the effect of antidepressants on Trx, TrxR, and Txnip in HT22 cells. HT22 cells were treated with vehicle or SSRI antidepressant fluoxetine at concentrations of 0.31, 0.62, 1.25 and 2.5 μM concentrations for 5 days. Protein levels of Trx, TrxR, and Txnip were measured by western blotting. We found that chronic treatment with fluoxetine at 1.25 and 2.5 μM significantly

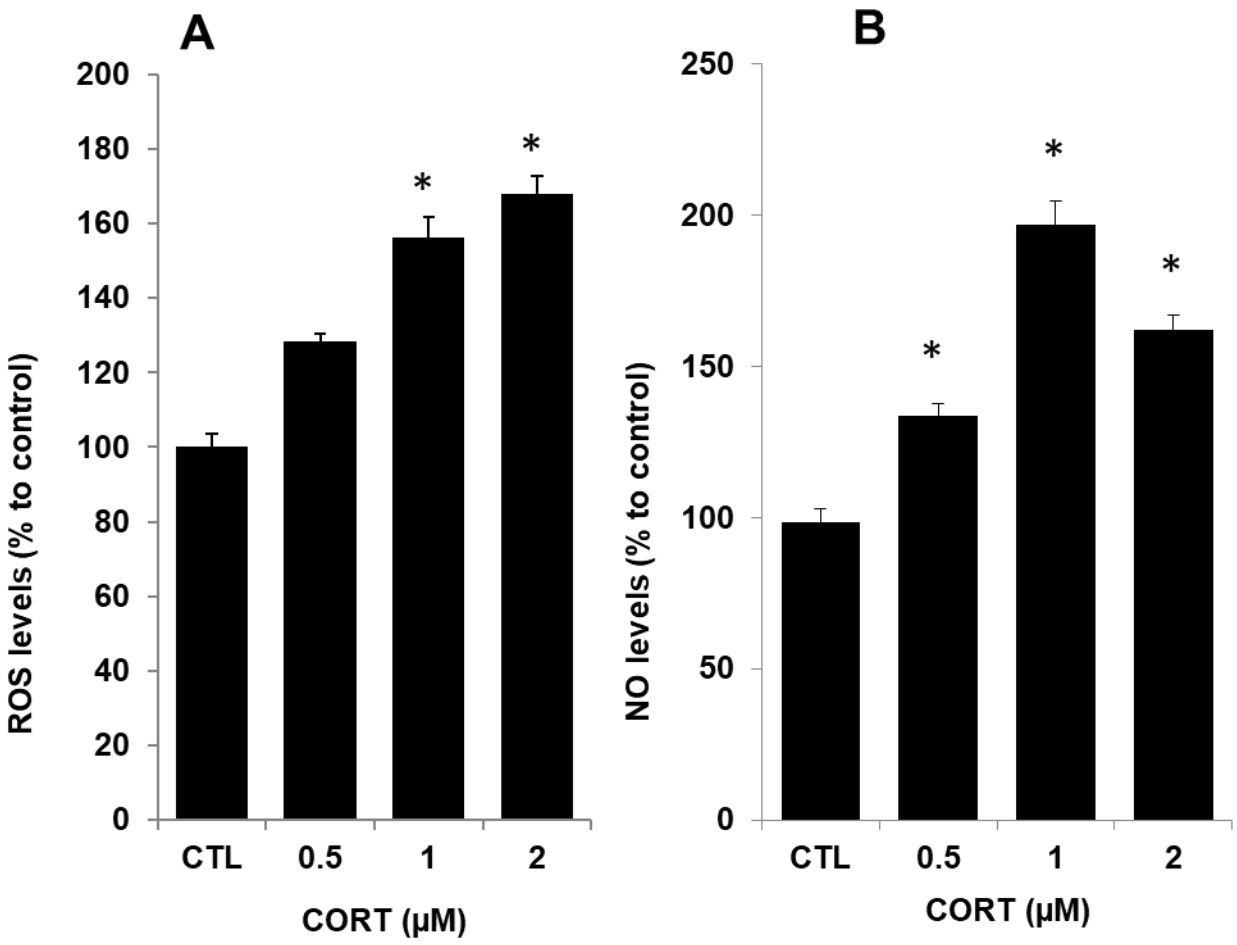


Fig. 13: The effect of corticosterone on reactive oxygen species (ROS) and nitric oxide (NO) levels in HT22 cells. HT22 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. ROS was measured by H_2DCFDA method. (A). NO was measured by Griess reagent method (B). Data are displayed as mean ± SEM (N =4) * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

increased the Trx protein levels ($p<0.05$, N=4-6), and treatment with fluoxetine at 0.62, 1.25 and 2.5 µM also significantly increased TrxR protein levels ($p<0.05$, N=4-6) (**Fig. 14 A and 14 B**). However chronic treatment with fluoxetine has no effect on Txnip levels (**Fig. 14 C**). HT22 cells were also treated with SNRI antidepressant venlafaxine at concentrations of 1.25, 2.5, 5 and 10 µM for 5 days. We also found that chronic treatment with venlafaxine at 5 and 10 µM significantly increased Trx and TrxR protein levels in HT22 cells ($p<0.05$, N=4-6) (**Fig. 15 A and 15 B**), but chronic treatment with venlafaxine has no effect on Txnip levels (**Fig. 15 C**).

3.3.2. To measure the effect of antidepressants on H_2O_2, GSNO and CORT induced sulfenylation and nitrosylation in HT22 cells.

Because Trx can reverse protein cysteine sulfenylation and nitrosylation, we measured the effect of antidepressants on sulfenylation and nitrosylation in HT22 cells. First, we measured the effect of antidepressants on H_2O_2-induced sulfenylation and GSNO-induced nitrosylation in HT22 cells. Cells were treated with vehicle, fluoxetine at 1.25 µM or venlafaxine at 5 µM for 5 days, and then incubated with H_2O_2 at 150 µM or GSNO at 40 µM for 30 min. We found that although treatment with fluoxetine or venlafaxine alone has no effect on sulfenylated protein levels, both fluoxetine and venlafaxine treatment significantly inhibited H_2O_2-increased sulfenylated protein levels ($p<0.05$, N=6) (**Fig. 16**). Although treatment with fluoxetine or venlafaxine has no effect on nitrosylated protein levels, both fluoxetine and venlafaxine treatment also inhibited GSNO-increased nitrosylated protein levels ($p<0.05$, N=6) (**Fig. 17**). These findings

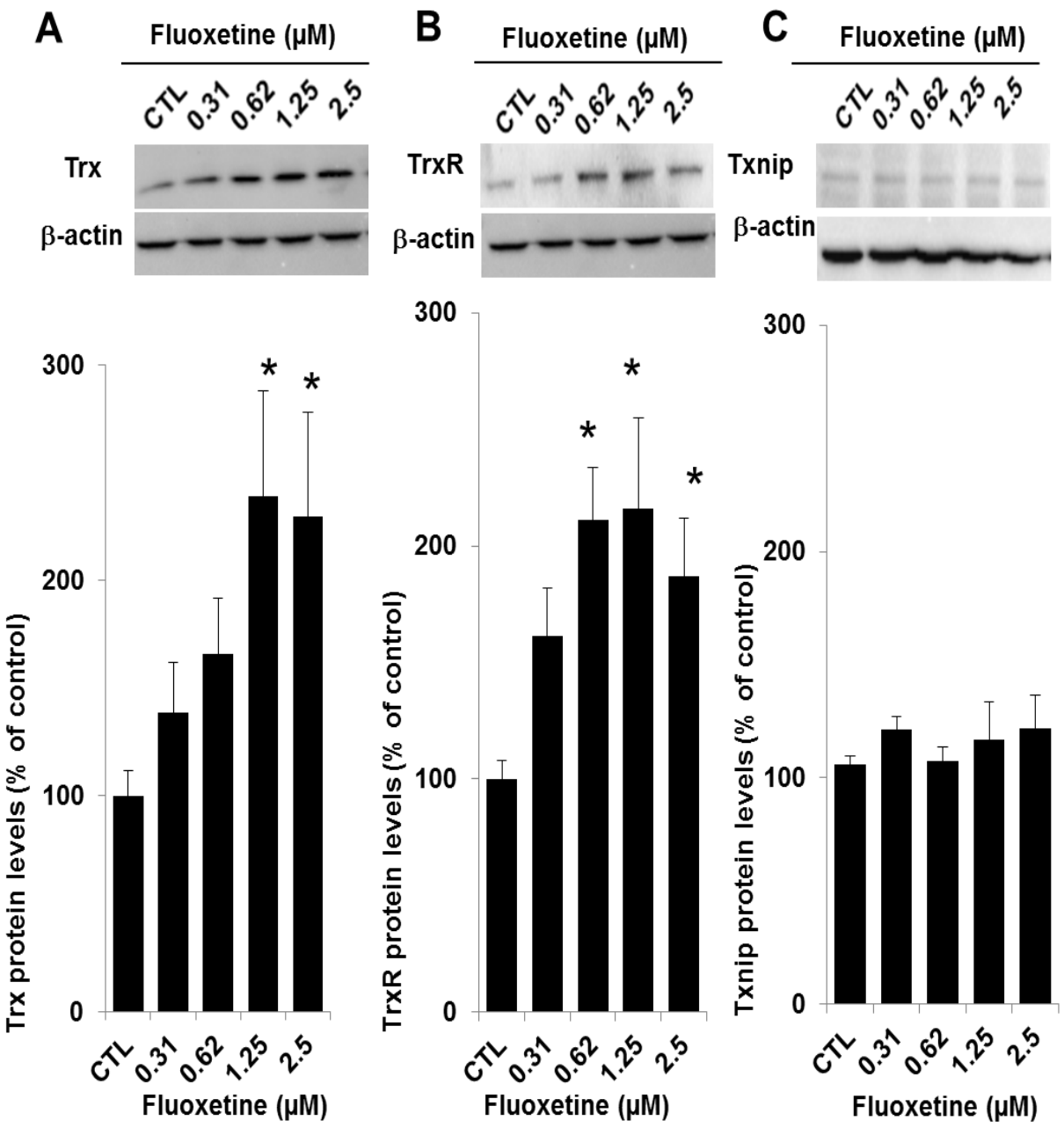


Fig. 14: The effect of fluoxetine on Trx, TrxR, and Txnip protein levels in HT22 cells. HT22 cells were treated with vehicle (CTL) or fluoxetine for 5 days. Protein levels of Trx (A), TrxR (B), and Txnip (C) were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =4-6). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

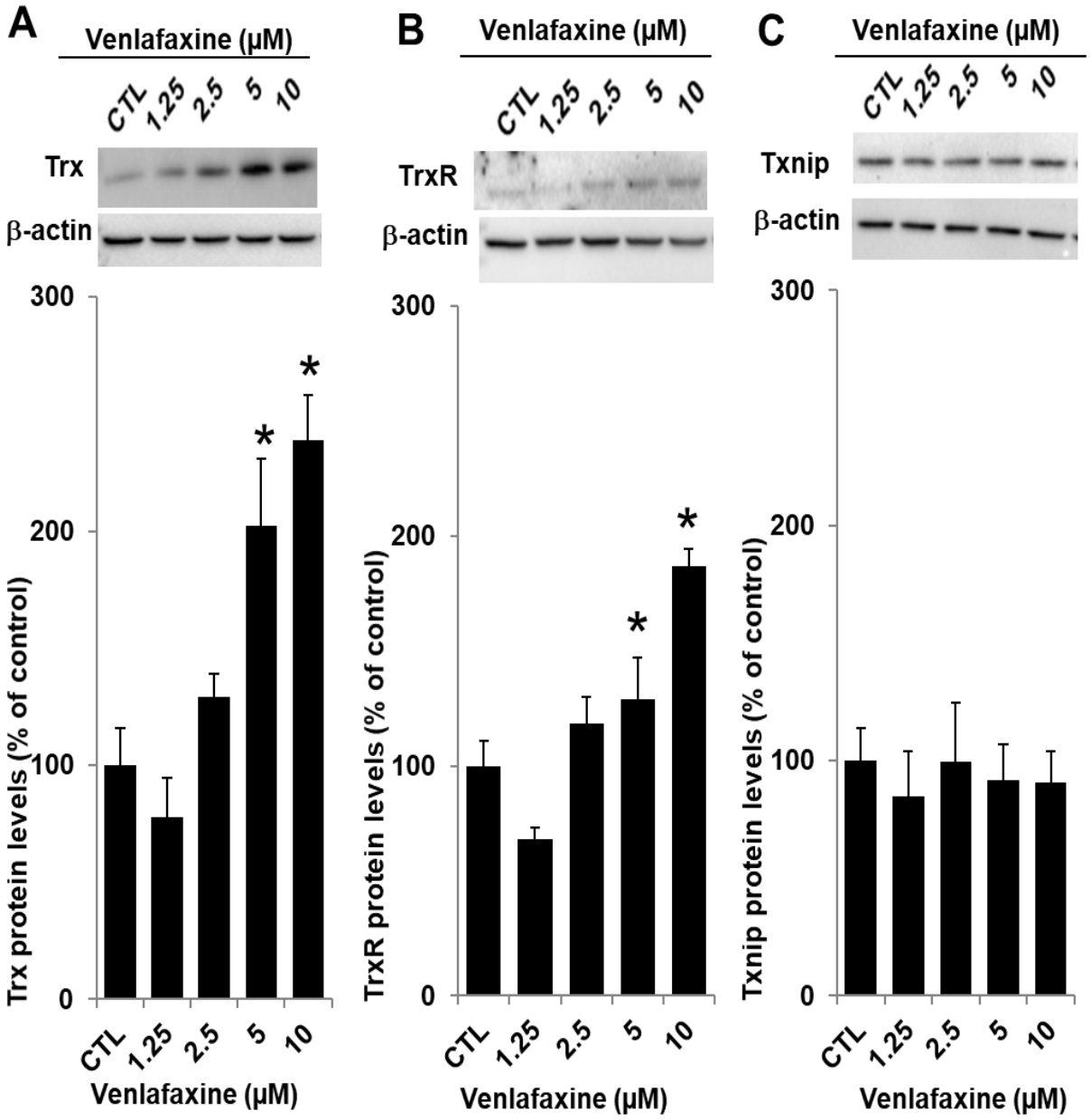


Fig. 15: The effect of venlafaxine on Trx, TrxR, and Txnip protein levels in HT22 cells. HT22 cells were treated with vehicle (CTL) or venlafaxine for 5 days. Protein levels of Trx (A), TrxR (B), and Txnip (C) were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =4-6). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

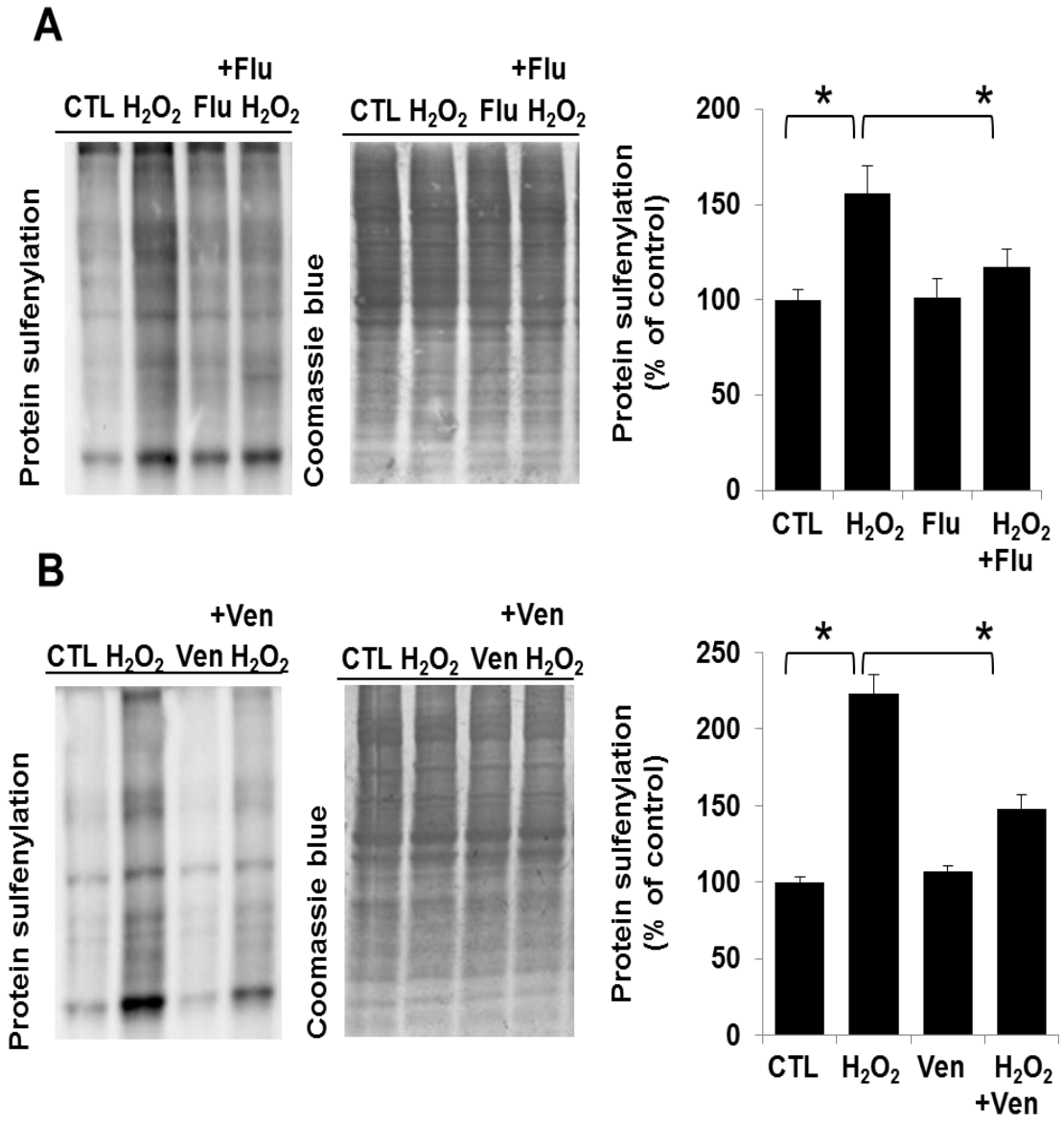


Fig. 16. The effect of fluoxetine and venlafaxine on H_2O_2-induced protein sulfenylation in HT22 cells. Cells were treated with vehicle (CTL) or fluoxetine (A) or venlafaxine (B) for 5 days. HT22 cells were treated with H_2O_2 for 30 min. Sulfenylated protein was measured by dimedone conjugation followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N =6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

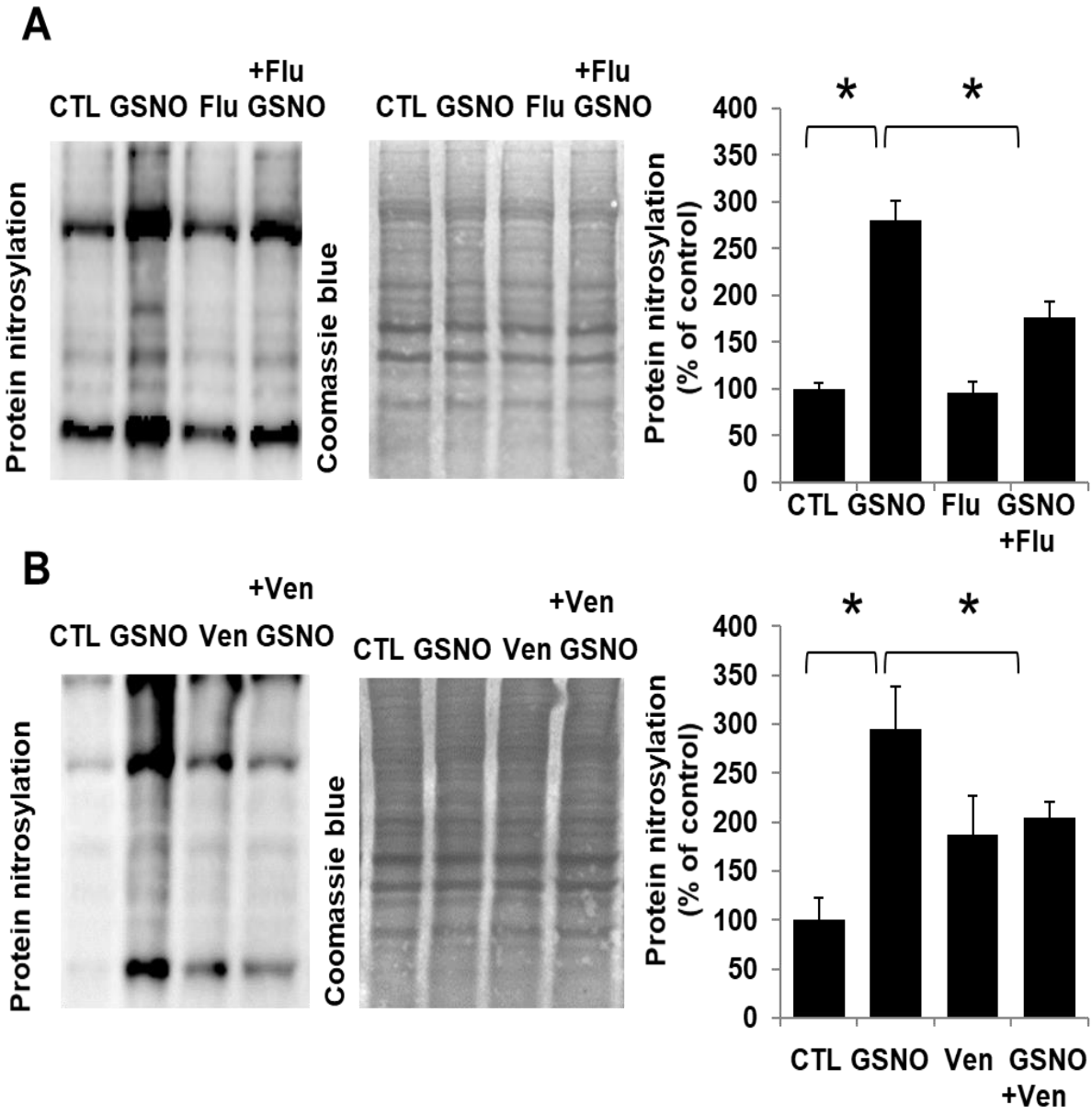


Fig. 17. The effect of fluoxetine and venlafaxine on GSNO-induced protein nitrosylation in HT22 cells. Cells were treated with vehicle (CTL) or fluoxetine (A) or venlafaxine (B) for 5 days. HT22 cells were treated with GSNO for 30 min. Nitrosylated protein was measured by biotin switch method followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N =6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

suggest that chronic treatment with antidepressants inhibited oxidants-induced protein cysteine sulfenylation and nitrosylation. Second, we measured the effect of antidepressants on CORT-induced protein cysteine sulfenylation and nitrosylation in HT22 cells. Cells were treated with vehicle, CORT at 1 μM concentration, fluoxetine at 1.25 μM or venlafaxine at 5 μM, CORT plus fluoxetine at 1.25 μM and CORT plus venlafaxine at 5 μM concentrations for 5 days. We verified that chronic CORT treatment significantly increased sulfenylated and nitrosylated protein levels. Although treatment with fluoxetine or venlafaxine alone had no effect on sulfenylated and nitrosylated protein levels, this treatment significantly inhibited CORT-increased sulfenylated protein levels **(Fig. 18 A**) and CORT-increased nitrosylated protein levels (**Fig. 18 B**).

3.4. To determine the effect of CORT on Trx system in microglia

3.4.1. To measure the effect of chronic CORT treatment on Trx, TrxR and Txnip protein levels in mouse N9 microglia and primary cultured mouse microglia.

First, the effect of chronic CORT treatment on protein levels of Trx, TrxR, and Txnip was measured in N9 cells. Cells treatment was done with CORT at concentrations of 0.5, 1 and 2 μM for 5 days, followed by measurement of protein levels of Trx, TrxR, and Txnip by western blotting. We didn't find the effect of chronic CORT treatment on Trx and TrxR protein levels but increased Txnip protein levels at 0.5, 1 and 2 μM concentrations ($p<0.05$, N=6) (**Fig. 19**).

Second, we verified the Trx, TrxR, and Txnip protein levels upon chronic CORT treatment in primary cultured mouse microglia. Cells were treated with CORT at 1 μM

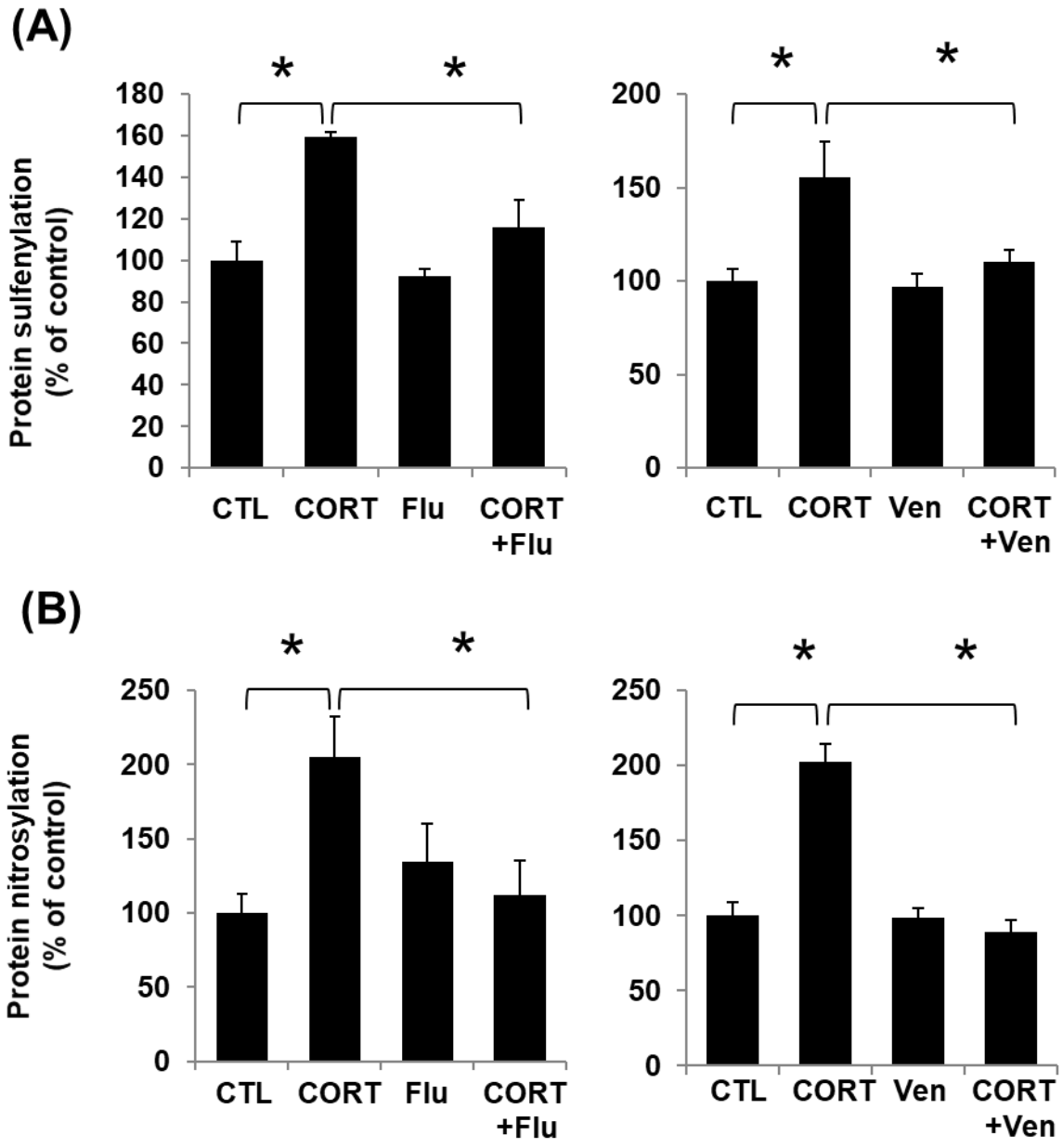


Fig. 18. The effect of fluoxetine and venlafaxine on CORT induced protein sulfenylation and nitrosylation in HT22 cells. Cells were treated with CORT at 1 μM, fluoxetine at 1.25 μM or venlafaxine at 5 μM, and CORT plus fluoxetine or venlafaxine for 5 days. Sulfenylated protein was measured by dimedone conjugation followed by immunoblotting analysis (A). Nitrosylated protein was measured by biotin switch method followed by immunoblotting analysis (B). Band sigal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N =6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

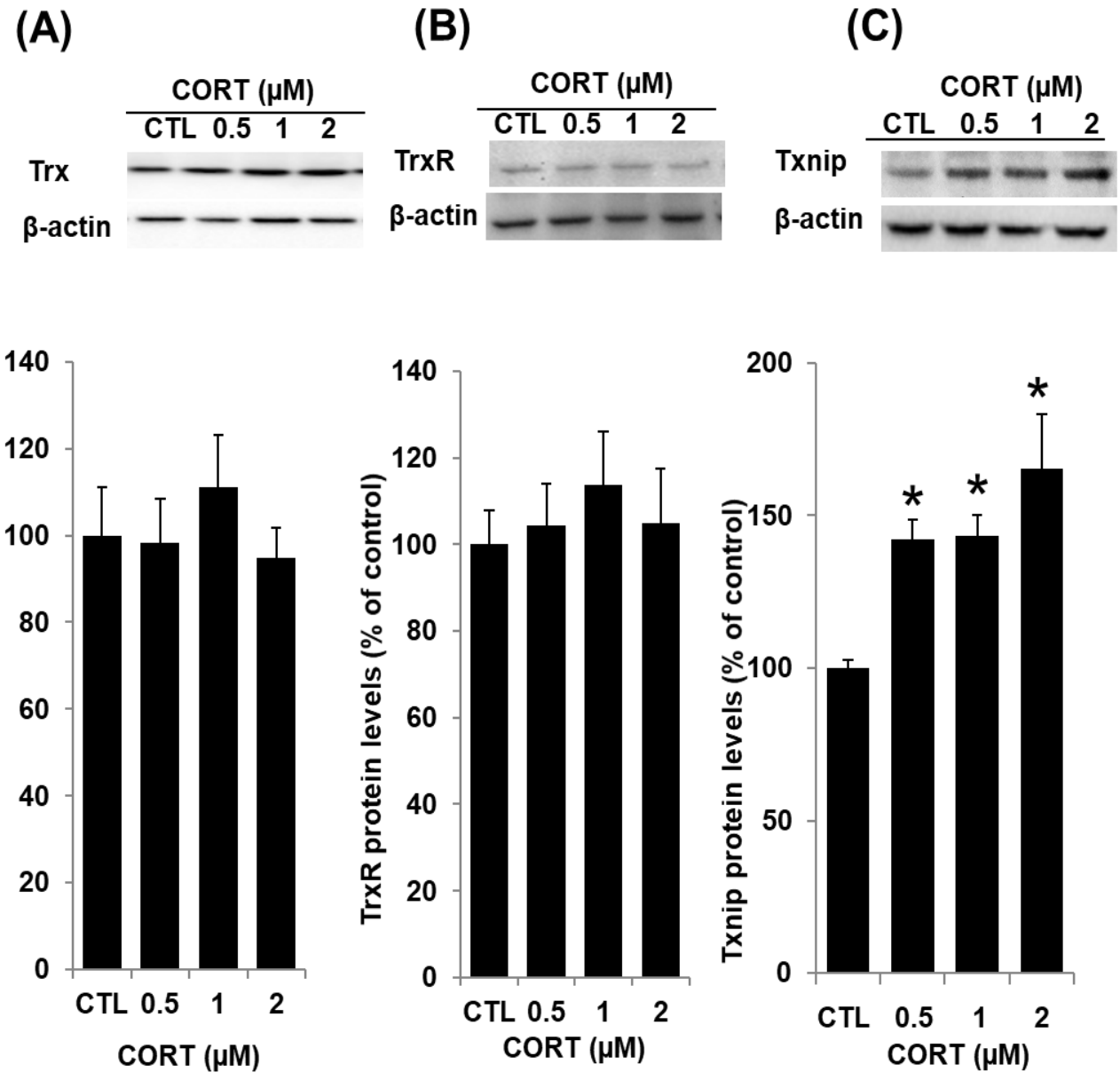


Fig. 19: The effect of corticosterone on Trx, TrxR, and Txnip protein levels in N9 cells. N9 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Protein levels of Trx (A), TrxR (B), and Txnip (C) were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N=6). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

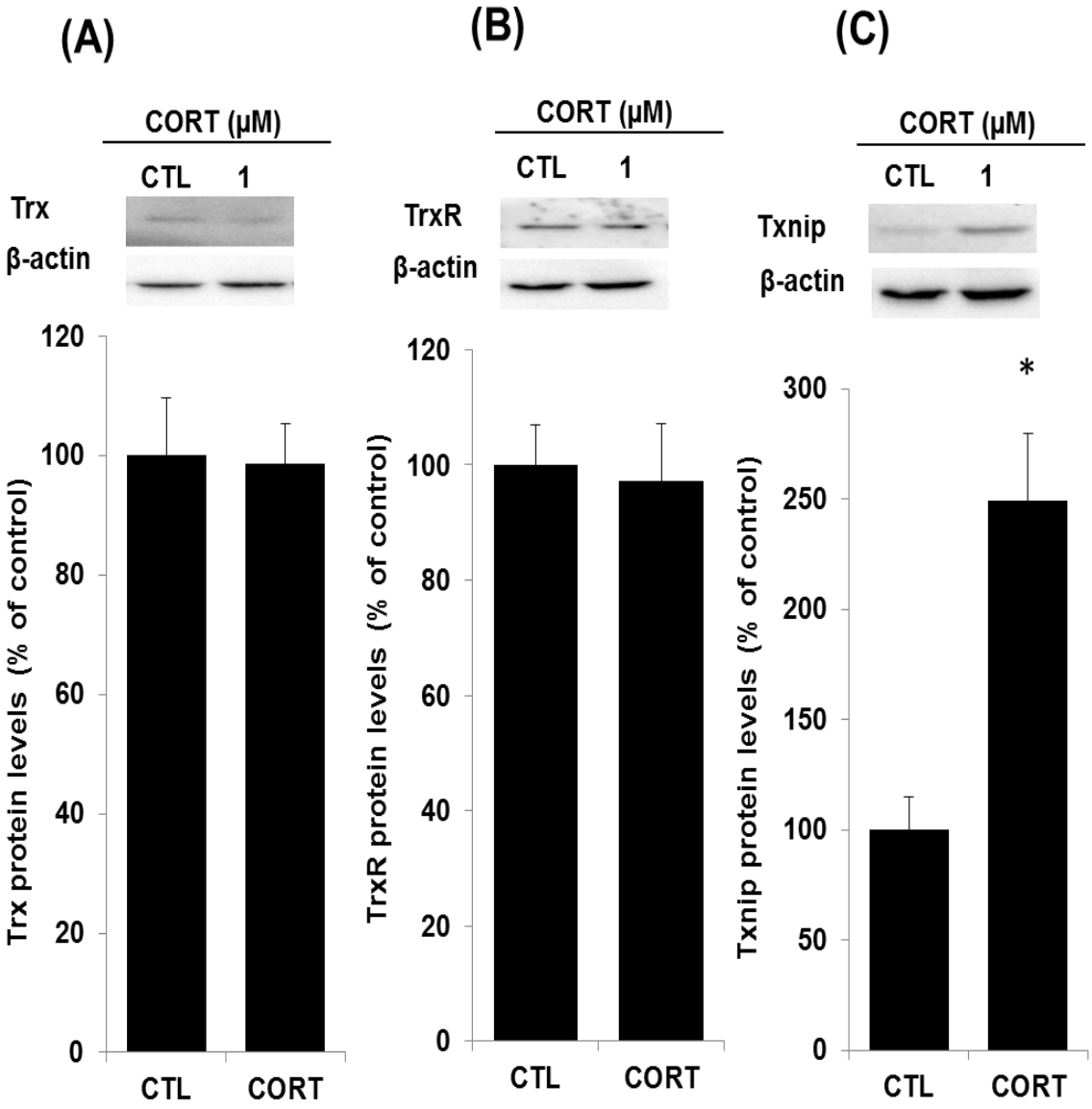


Fig. 20: The effect of corticosterone on Trx, TrxR and Txnip protein levels in primary cultured microglia. Primary cultured microglia were treated with vehicle (CTL) or corticosterone (CORT) at 1 μM concentration for 5 days. Protein levels of Trx (A), TrxR (B), and Txnip (C) were measured with Trx, TrxR, and Txnip antibodies using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =4). * indicates $p<0.05$ when compared to controls determined by T-test.

for 5 days. We found increased Txnip protein levels, but had no effect on Trx and TrxR protein levels ($p<0.05$, N=4) **(Fig. 20).**

3.4.2. To measure the effect of chronic CORT treatment on Txnip/NLRP3 binding activity in N9 cells, and the effect of chronic stress on Txnip/NLRP3 binding activity in mouse hippocampus and frontal cortex.

Txnip in microglia can bind to NLRP3 and activate NLRP3 inflammasome signaling. First, we measured the effect of chronic CORT treatment on NLRP3 protein levels. Lipopolysaccharide (LPS) is an endotoxin and can upregulate NLRP3 [117]. LPS was used as a positive control. Cells were treated with vehicle or CORT at concentration 0.5, 1 and 2 μM for 5 days. For the positive control group, cells were treated with vehicle or LPS at 500 ng/mL concentration for 4 h. As shown in **Fig. 21 A and 21 B**, treatment with LPS significantly increased NLRP3 protein levels ($p<0.05$, N=3), but treatment with CORT had no effect on NLRP3 protein levels.

Second, we measured the effect of chronic CORT treatment on Txnip/NLRP3 binding activity in N9 cells. N9 cells were treated with CORT at 1 μM for 5 days. Protein extract was immunoprecipitated with NLRP3 antibody. Then pulled down proteins were analyzed with Txnip antibody using western blotting. The membrane blot was then re-probed with NLRP3 antibody. As shown in **Fig. 22A,** chronic CORT treatment significantly increased Txnip/NLRP3 binding ($p<0.05$, N=15).

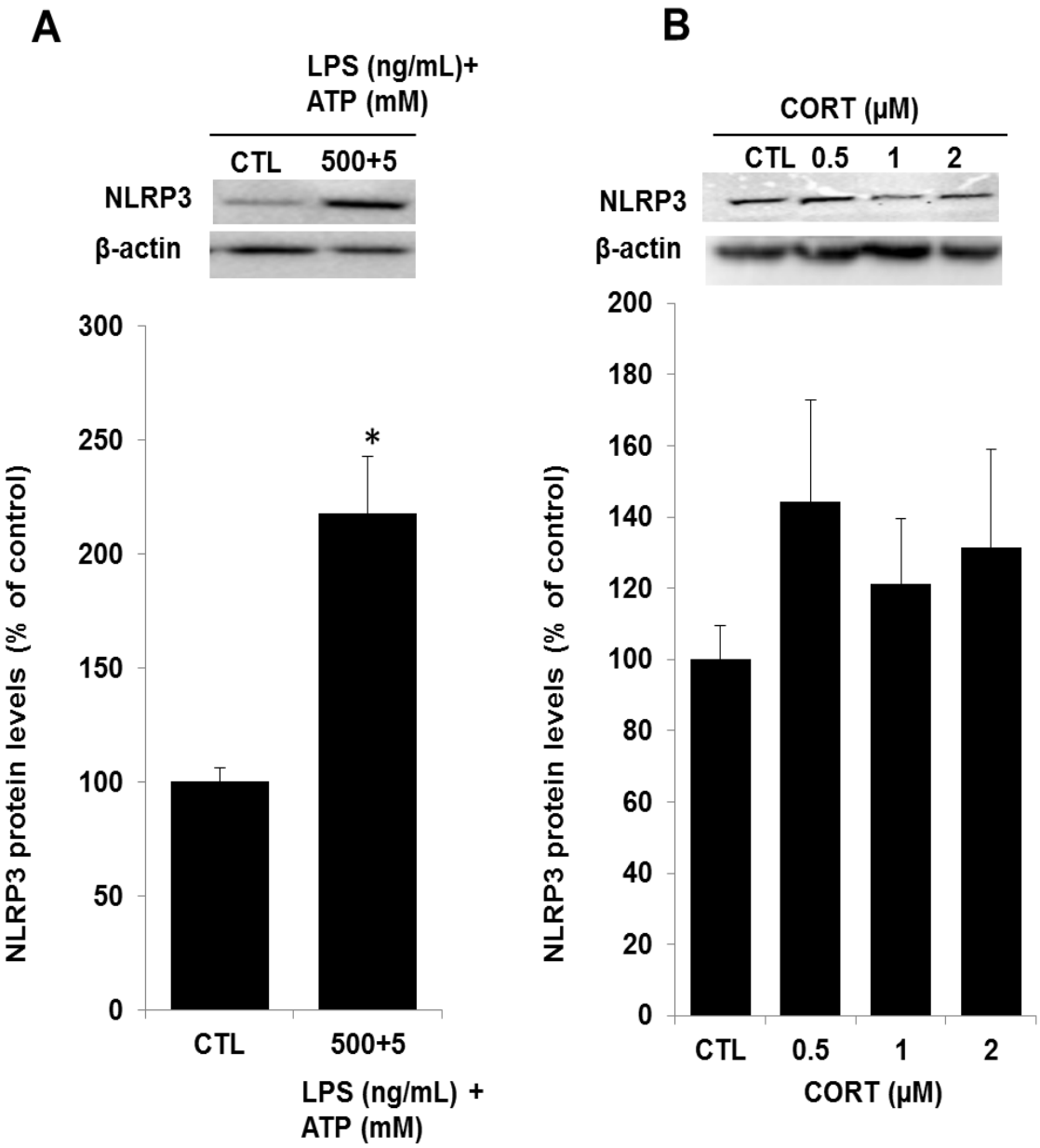


Fig. 21: The effect of corticosterone on NLRP3 protein levels in N9 cells. N9 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days (B). N9 cells were treated with LPS for 4 h followed by ATP treatment for 30 min. (A). Protein levels of NLRP3were measured with NLRP3 antibody using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =3-6). * indicates $p<0.05$ when compared to controls determined by T-Test (A) and one-way ANOVA followed by Tukey's post-hoc analysis (B).

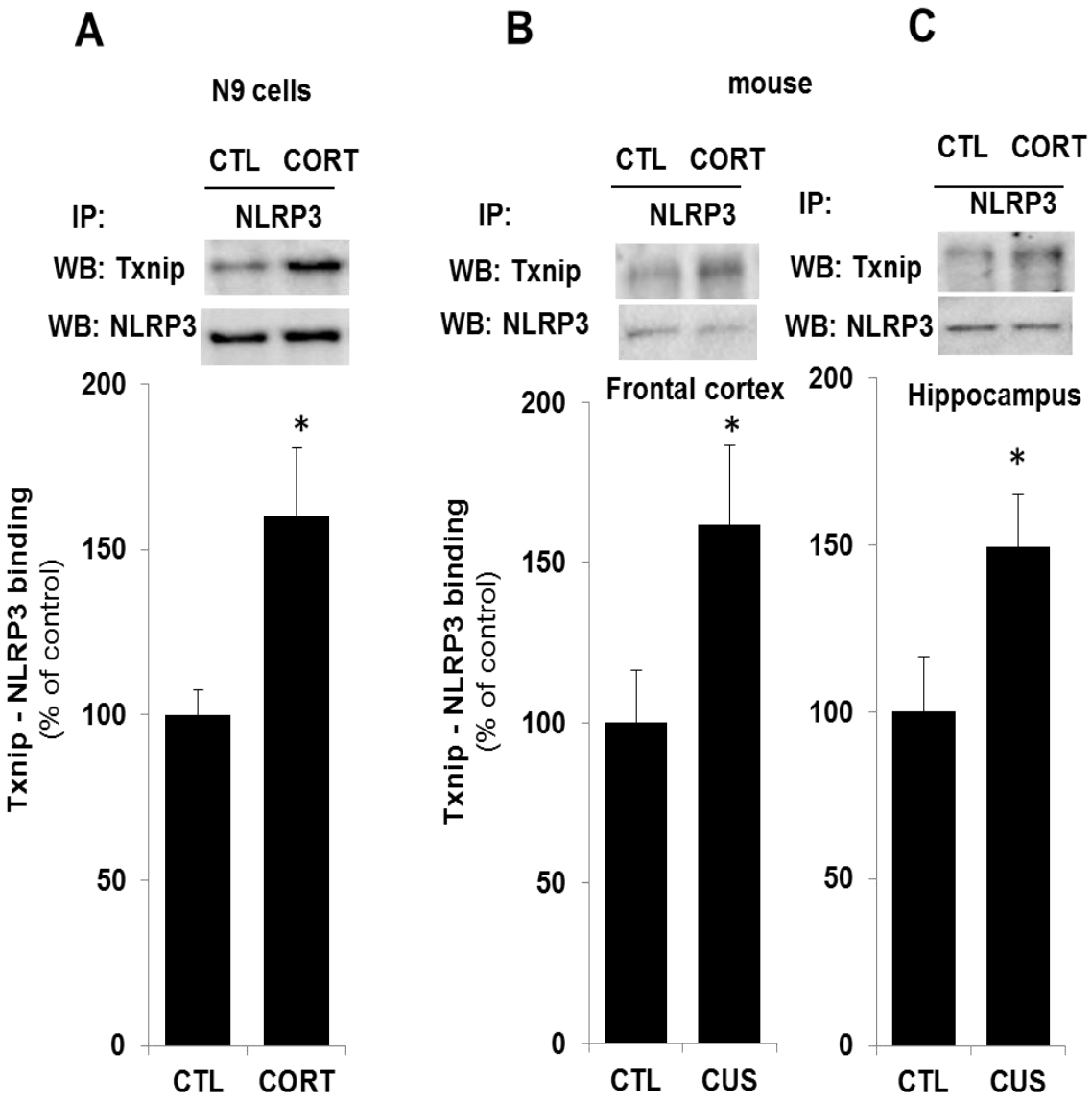


Fig. 22: The effect of corticosterone on NLRP3/Txnip binding in N9 cells and in frontal cortex and hippocampus of stressed mice. N9 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. NLRP3/Txnip binding was measured using co-immunoprecipitation followed by immunoblotting analysis in N9 cells treated with CORT (A), frontal cortex (B), and hippocampus (C) of chronic stressed mice for 28 days. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =10-15). * indicates $p<0.05$ when compared to controls determined by T-test.

Third, we measured the effect of chronic stress on Txnip/NLRP3 binding activity in mouse hippocampus and frontal cortex. Mouse brain tissue was provided by the graduate student Hong Zhou in our laboratory. We found that NLRP3/Txnip binding was significantly increased in the hippocampus and frontal cortex of mice exposed to chronic unpredictable stress for 4 weeks when compared to controls ($p<0.05$, N=12-13) (**Fig. 22B and 22C)**.

3.4.3. To measure the effect of chronic CORT treatment on caspase-1 activation and IL-1β release in N9 cells.

NLRP3 inflammasome upon activation can cleave procaspase-1 to the active caspase-1 and increase the release of IL-1β. First, we treated N9 cells with 500 ng/mL LPS for 4 h followed by 5 mM ATP treatment for 30 min. Procaspase-1 protein levels in cell extracts were measured by immunoblotting analysis using anti-caspase-1 antibody. We have found that LPS plus ATP treatment significantly decreased the procaspase-1 protein levels in cell extracts. Then we treated N9 cells with CORT at 0.5, 1 and 2 μM concentrations for 5 days. Procaspase-1 protein levels were analyzed by immunoblotting in cell extracts using anti-procaspase-1 antibody. We found that CORT at 0.5, 1 and 2 μM concentrations decreased the procaspase-1 protein levels **(Fig. 23).**

Second, we measured the effect of chronic CORT treatment on caspase-1 activation and IL-18 release. Cells were treated with CORT at concentrations of 0.5, 1 and 2 μM for 5 days. We found that although treatment with CORT at 0.5 μM had no effect on caspase-1 activity, treatment with CORT at 1 and 2 μM significantly increased caspase-1 activity in

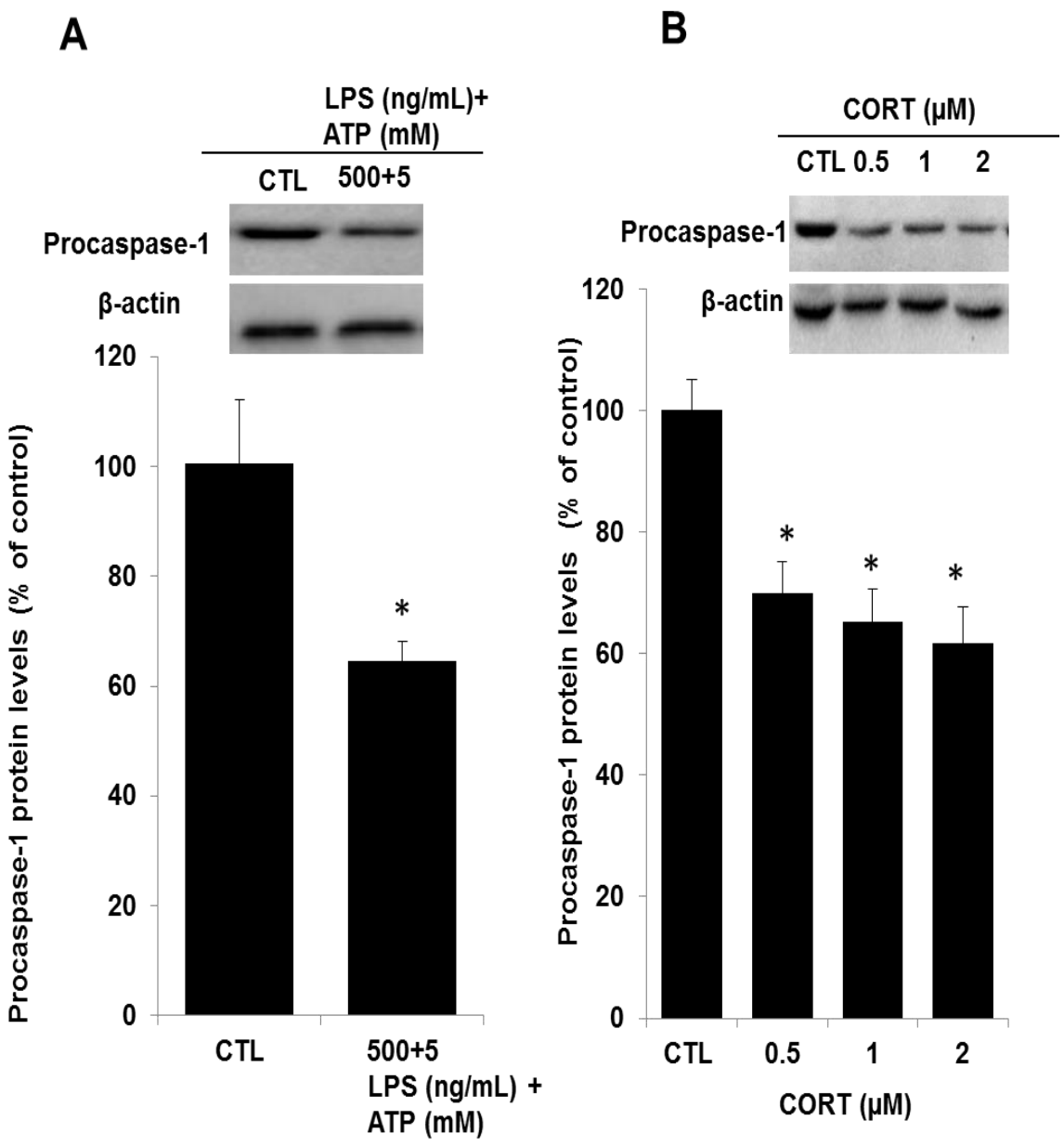


Fig. 23. The effect of corticosterone on procaspase-1 protein levels in N9 cells. N9 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days (B). N9 cells were treated with LPS for 4 h followed by ATP treatment for 30 min. (A). Protein levels of procaspase-1were measured with procaspase-1 antibody using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to controls by T-test (A) and determined by one-way ANOVA followed by Tukey's post-hoc analysis (B).

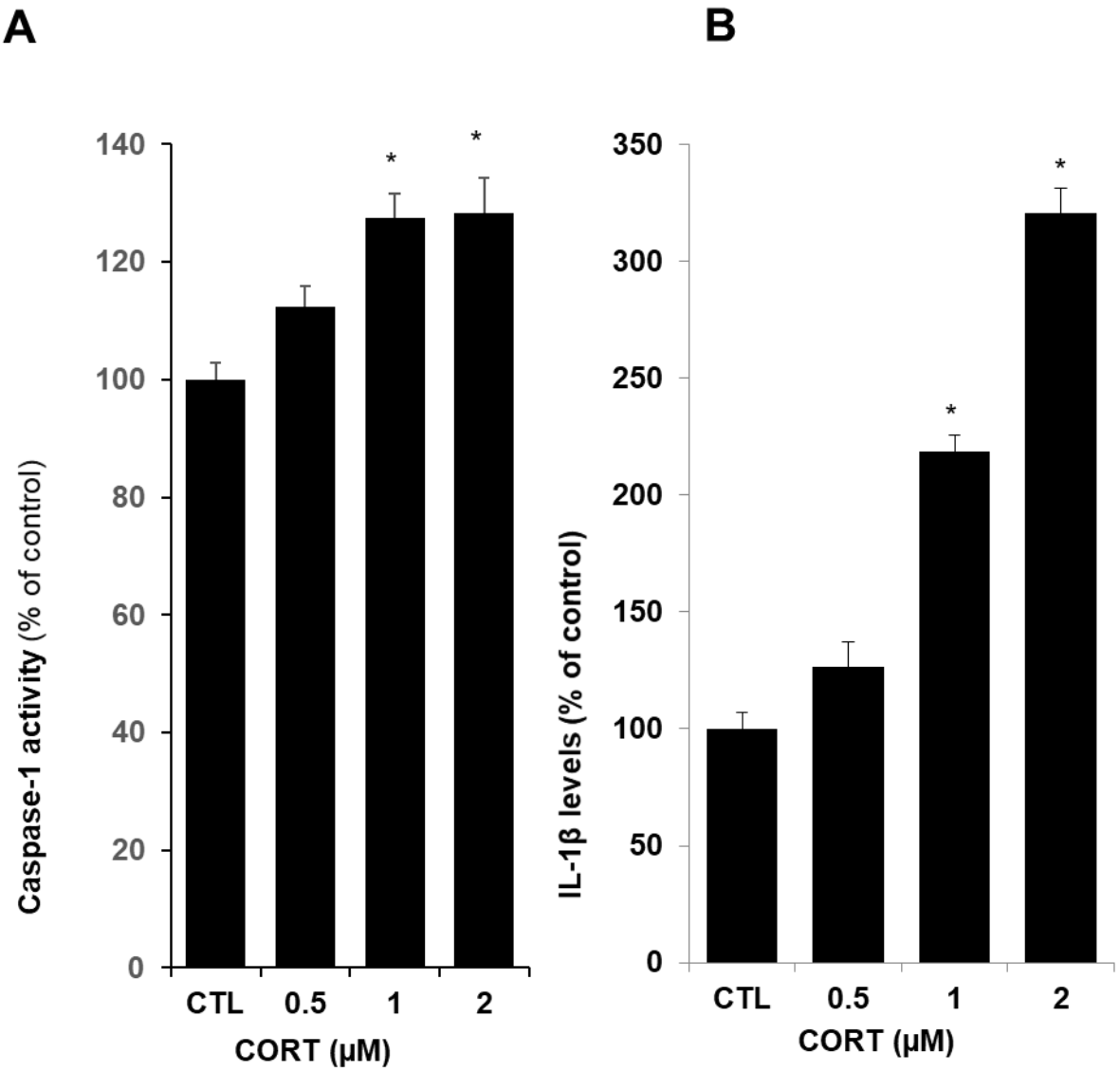


Fig. 24: The effect of corticosterone on caspase-1 activity and protein levels of IL-1 β in N9 cells. N9 cells were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. N9 cells were treated with LPS alone for 4 h and LPS for 4 h and ATP for 30 min. Activity of caspase-1 was measured using caspase-1 substrate (Z-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin). Fluorescence was measured at 400 and 505 nm (A). Protein levels of IL-1β were measured using ELISA kit (B) Data are displayed as mean ± SEM (N =6-8). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

N9 cells ($p<0.05$, N=6-8). We also found that treatment with CORT at 1 and 2 μM significantly increased IL-1β levels in cultured medium ($p<0.05$, N=6-8) (**Fig. 24 A and 24 B)**, suggesting that CORT treatment can activate caspase-1 and release IL-1β.

Third, to determine if Txnip mediates CORT-increased caspase-1 activation and IL-1β release, we analyzed the effect of Txnip knockout on CORT-increased caspase-1 activation and IL-1β release in N9 cells. Txnip gene was knocked out by CRISPR/Cas9 technology as previously described. Scrambled sgRNA was used as control. As shown in **Fig. 25**, Txnip protein levels were significantly knocked out in N9 cells transfected with Txnip sgRNAs when compared to wild-type cells and cells transfected with scramble sequence. We also found that although chronic treatment with CORT at 1 μM increased caspase-1 activity in cells transfected with scramble sequence ($p<0.05$, N=6-8), this treatment had no effect on caspase-1 activity in cells transfected with Txnip sgRNA, suggesting that Txnip knockout inhibited caspase-1 activity (**Fig. 26 A**). We also found that chronic treatment with CORT at 1 μM inhibited CORT-increased IL-1β levels in culture medium (**Fig. 26 B**). These results suggest that Txnip may mediate CORT-activated caspase-1 activity and CORT-increased IL-1β release.

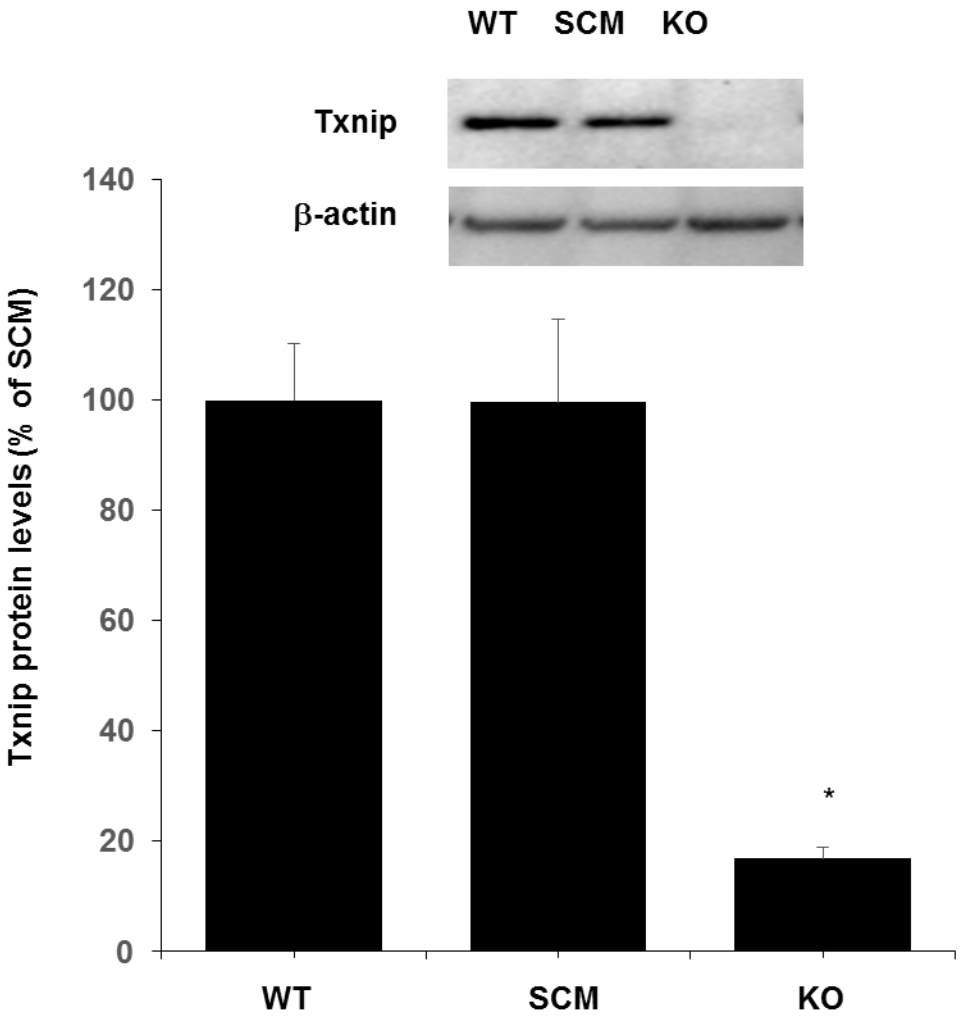


Fig. 25. Txnip gene knockout in N9 cells using CRISPR-Cas9 technology. Lenti-vectors containing Txnip sgRNAs/CRISPR/Cas9 (KO) or scrambled sgRNAs/CRISPR/Cas9 (SCM) were transfected into N9 cells. Txnip protein levels were measured by immunoblotting analysis. Data are displayed as mean ± SEM (N = 3). * indicates $p < 0.05$ when KO group was compared to WT and SCM groups determined by one-way ANOVA followed by Tukey's post-hoc analysis.

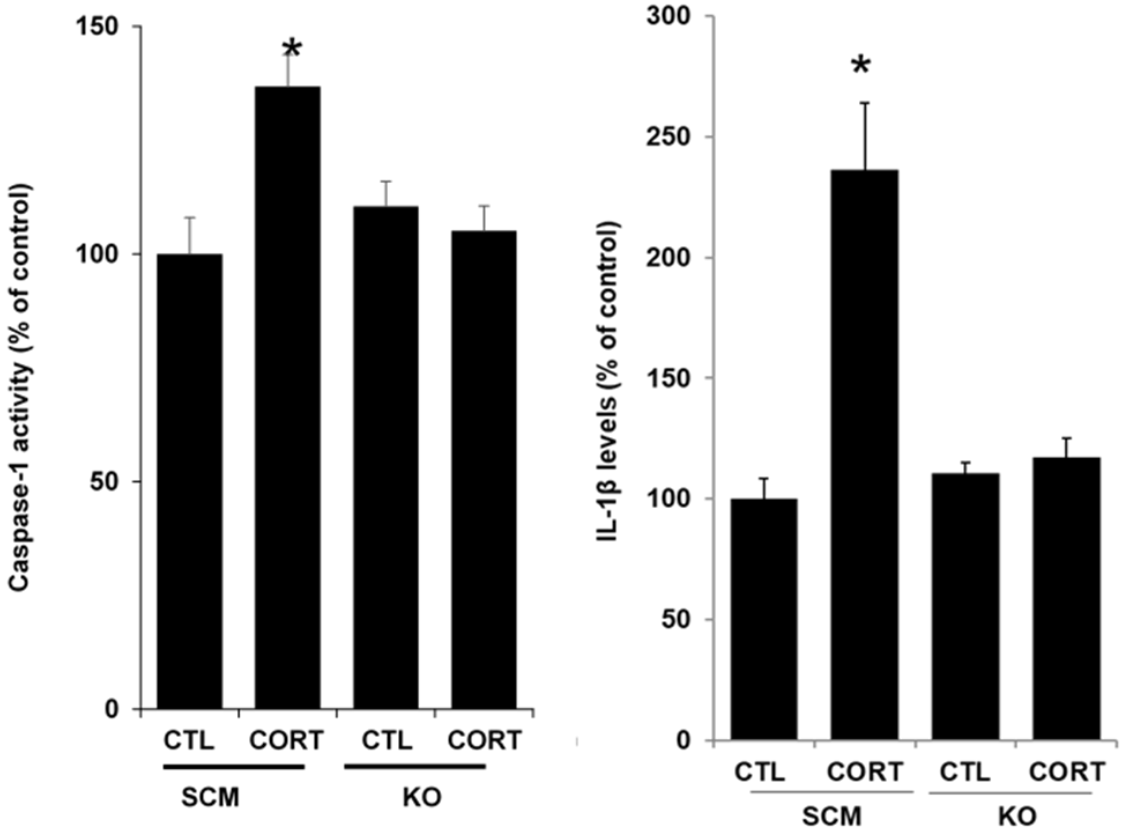


Fig. 26: The effect of corticosterone on caspase-1 activity and IL-1β levels in Txnip KO N9 cells. N9 cells (scrambled and Txnip KO) were treated with vehicle (CTL) or corticosterone (CORT) for 5 days. Activity of caspase-1 was measured using caspase-1 substrate (Z-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin). Fluorescence was measured at 400 and 505 nm (A). Protein levels of IL-1β were measured using ELISA kit (B). Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

CHAPTER 4: DISCUSSION

4.1. The effect of glucocorticoids on Trx, TrxR, and Txnip:

Upregulation of Txnip by CORT: In our study, we have found that although chronic treatment with CORT has no effects on Trx and TrxR protein levels in cultured HT22 mouse hippocampal cells and primary cultured cerebral cortical neurons, this treatment upregulated the protein levels of Txnip in both HT22 cells and primary cultured cerebral cortical neurons. Since Cys-247 of Txnip can bind to Cys-32 of Trx in cytosolic and mitochondrial compartments, and act as an endogenous inhibitor of Trx, these results suggest that upregulation of Txnip protein levels may further inhibit Trx enzyme activity. Previously our laboratory exposed mice to chronic unpredictable stress for 28 days; and found that chronic unpredictable stress reduced exploratory, increased anhedonia and induced despair behaviours in mice, suggesting that chronic stress can cause depressive-like behaviours [98]. Further our laboratory also found that chronic unpredictable stress increased Txnip protein levels in mouse hippocampus and frontal cortex[98]. Chronic unpredictable stress was also found to increase Txnip protein levels in rat hippocampus[98]. Stress in long-term can increase serum CORT levels in rodents[118]. These findings together suggest that chronic stress may increase CORT, resulting in the increase of Txnip expression and that increased Txnip may contribute to depressive behaviours.

The role of GR in CORT-upregulated Txnip: We have also shown that CORT treatment for 5 days increased mRNA levels of Txnip in cultured HT22 cells [119] . Chronic stress was also found to increase Txnip mRNA levels in rat hippocampus[120]. These findings indicate that CORT-increased Txnip gene transcription may contribute to increased Txnip protein levels. It has also been reported that Txnip gene promoter

contains a functional glucocorticoid response element from -940 to -764 and 6-36 hours long treatment with GR agonist dexamethasone increased Txnip mRNA levels in WEHI7.2 T-cell lymphoma line and mouse thymocytes [121], suggesting that glucocorticoid may bind to glucocorticoid response element, subsequently turning on Txnip gene expression.

CORT can target both GR and MR. Previously it has been shown that GR inhibitor RU486 completely blocked [^{3}H]-CORT binding in HT22 cells that, suggesting that GR but not MR are mainly expressed in HT22 cells [122] . We found that RU-486 completely blocked CORT-increased Txnip protein levels in HT22 cells [115]. Our findings suggest that GR mediate CORT-upregulated Txnip in HT22 cells. Further, we also verified that RU486 blocked CORT-induced Txnip protein upregulation in primary cultured cerebral cortical neuronal cells. RU-486 was also found to decrease chronic stress-increased Txnip mRNA levels in rat hippocampus[120]. Cerebral cortex and hippocampus express both GR and MR[123,124]. GR and MR can form a heterodimer that mediates corticosteroid action[125]. Further investigation is required to determine if MR also plays a role in CORT and chronic stress-increased Txnip.

The possible roles of transcription factors Nrf2 in CORT-upregulated Txnip: It is interesting that Txnip gene also contains an antioxidant response element (ARE) in -1286 to -1276 in the gene promoter region. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that primarily binds to ARE sites and regulates ARE-driven gene expression [126]. Nrf2 was also found to regulate Txnip gene expression [127] . It has been

reported that Txnip mRNA and protein levels were increased in the heart of Nrf2 knockout mice as compared to the heart of wild type mice. Nrf2 overexpression also inhibited Txnip mRNA and protein levels, while Nrf2 knockdown increased Txnip mRNA and protein levels in cultured rat H9C2 cardiomyocyte cells [127] . Both *in vivo* and *in vitro* studies suggest that Nrf2 can suppress Txnip expression. When Txnip promoter region was cloned into luciferase reporter vector and transfected into H9C2 cells, co-transfection with Nrf2 expression plasmid was found to inhibit luciferase reporter expression, but co-transfection with Nrf2 siRNA increased luciferase reporter expression. Further, ARE mutation in Txnip promoter region was also found to increase luciferase reporter expression. The ChIP-qPCR analysis showed that Nrf2 can bind to Txnip ARE site [127] . These findings suggest that Nrf2 can negatively regulate Txnip gene expression by binding to ARE site.

Previous evidences indicate that chronic stress and CORT treatment inhibit Nrf2. For example, it has been reported that rats kept in isolation condition for 21 days followed by immobilization stress for 30 min showed a decreased nuclear Nrf2 protein levels in hippocampal and prefrontal cortical [128] . Nrf2 nuclear protein levels were also decreased in the hippocampus of rats exposed to chronic restraint stress when compared to controls [40]. Nrf2 mRNA and protein levels were also decreased in hippocampus and frontal cortex of rats exposed to chronic unpredictable stress[40]. Further chronic treatment with CORT at 35 mg/mL in drinking water for 4 weeks also decreased Nrf2 protein levels in mouse cortex and hippocampus [42]. These studies suggest that chronic stress and chronic CORT treatment can decrease Nrf2 protein levels that may lead to increased Txnip gene

expression. These findings also suggest that CORT may increase Txnip gene expression not only by binding to GRE site but also by inhibiting Nrf2-mediated negative regulation of ARE site.

4.2. The role of Txnip in glucocorticoid-induced oxidative stress

Txnip is also known as vitamin D3-upregulated protein-1 or Trx-binding protein-2 and was originally cloned as vitamin D_3 target gene in HL-60 cells. Upregulation of Txnip can promote oxidative stress, inflammation, and apoptosis.

Binding of Txnip and Trx results in the inhibition of Trx reducing capability, subsequently increasing cysteine oxidative modification of proteins such as sulfenylation and nitrosylation. In the present study, we showed that chronic CORT treatment in HT22 cells increased sulfenylated and nitrosylated protein levels, suggesting that CORT can induce sulfenylation and nitrosylation. Recently our laboratory also found that mice exposed to chronic unpredictable stress showed enhanced sulfenylated and nitrosylated protein levels in the hippocampus and frontal cortex as compared to controls [98]. These results suggest that chronic stress may increase CORT, subsequently enhancing cysteine sulfenylation and nitrosylation, which may contribute to chronic stress-induced oxidative stress.

Cysteine residues in proteins are critical in regulating functions of many proteins such as redox homeostasis, metal binding, enzymatic catalysis, and signal transduction [86]. The highly nucleophilic character of cysteine makes them more susceptible to oxidative stress

as compared to other amino acids [86]. Protein cysteine oxidative modification can have several effects on protein targets in neurons. It has been reported that S-nitrosoglutathione, a nitric oxide (NO) donor increased the nitrosylation of vesicular acetylcholine transporter and vesicular glutamate transporters in the mouse brain. S-nitrosothiols also reduced [^{3}H] acetylcholine and [^{3}H] glutamate uptake in mouse brain. Treatment of reducing agent dithiothreitol reversed S-nitrosoglutathione-reduced uptake of [^{3}H] acetylcholine and [^{3}H] glutamate [87]. These findings suggest that nitrosylation of vesicular acetylcholine transporter and vesicular glutamate transporters can inhibit vesicular neurotransmitter uptake. GluA1 is a subunit of the glutamate AMPA receptor. It has been reported that treatment with S-nitrosoglutathione increased nitrosylation at Cys-875 of GluA1 subunit, while phosphorylation at Ser-831 of GluA1 subunit increased the conductance of the AMPA receptor in cultured human HEK cells. Nitric oxide synthases (NOS) are a group of enzymes that catalyze NO production. Neuronal NOS knockout was found to reduce GluA1 Ser-831 phosphorylation and channel conductance in HEK cells[88], suggesting that nitrosylation of GluA1 subunit of the AMPA receptor can enhance GluA1 Ser-831 phosphorylation-increased conductance of the AMPA channels. Using proteomic-based approach, Monika et al. identified a number of nitrosylated proteins in the cortical cultures of 21 days long restraint-stressed mice [129]. These proteins target include catenin delta CaMKII, Iqseq7, Shank, Gria1, glycogen synthase kinase-3β (GSK3β), and PSD-95 [129]. Because these proteins are mainly involved in regulating synaptic plasticity, this finding suggests that chronic stress may nitrosylate these proteins, further impairing neuroplasticity.

To investigate the role of Txnip on protein cysteine oxidative modifications induced by CORT, we further knocked out Txnip in HT22 cells, treated with CORT and analyzed if CORT enhanced protein nitrosylation and sulfenylation. Knocking out Txnip reversed chronic CORT-increased sulfenylated and nitrosylated protein levels in cultured HT22 cells. Our findings indicate that chronic CORT treatment upregulates Txnip and inhibit Trx reducing activity, subsequently causing accumulation of protein cysteine oxidative modification.

H_2O_2 and NO can attack cysteine residues and cause cysteine sulfenylation and nitrosylation [130,131]. Previous studies have consistently shown that chronic CORT treatment increased ROS and NO levels in cultured neuronal cells and animal brain [39,132,133]. In the present study, we have also verified that chronic CORT treatment significantly increased ROS and NO levels in cultured HT22 cells, which is consistent with the previous findings [28,33,133]. Our findings suggest that increased levels of cysteine sulfenylation and nitrosylation may result from either Txnip-induced inhibition of Trx reducing activity or overproduction of ROS and NO.

Antioxidant peroxiredoxin can reduce H_2O_2 and other peroxides, and play an important role in peroxide detoxification. Previously it has been reported that oxidized inactive form of peroxiredoxin and peroxidation end product MDA were increased in the hippocampus of rats subjected to social defeat stress for 3 days followed by chronic unpredictable stress for 4 weeks [128] , suggesting that chronic stress may inhibit peroxiredoxin reducing activity, and inducing accumulation of peroxides. Since Trx can

reduce peroxiredoxin from the oxidized inactive state to the reduced active form, this study, together with our finding, suggests that chronic CORT treatment may increase Txnip, then inhibit Trx reducing activity, and increase the accumulation of oxidized inactive form of peroxiredoxin. This mechanism may also contribute to the oxidative stress process induced by chronic stress and chronic CORT treatment.

In summary, we found that chronic CORT treatment increased mRNA and protein levels of Txnip in cultured neuronal cells. CORT treatment also increased nuclear and cytoplasmic levels of Txnip. GR inhibitor RU-486 blocked the CORT-upregulated Txnip protein levels. We also found that protein cysteine sulfenylation and nitrosylation were increased by chronic CORT treatment and that knocking out Txnip inhibited CORT-induced sulfenylation and nitrosylation. Our findings suggest that chronic CORT treatment can upregulate Txnip in neuronal cells, resulting in Trx inhibition that facilitates protein cysteine oxidative modification, which contributes to stress-induced oxidative stress process.

4.3. The effect of antidepressants on Trx antioxidant system

Many studies have shown that antidepressants produced the neuroprotective effect against oxidative stress [134–136]. Since our laboratory found that chronic stress and stress hormone CORT treatment increased Txnip protein levels and protein cysteine oxidative modifications, in the present study, we also analyzed the effect of antidepressants on Trx, TrxR, and Txnip protein levels and protein cysteine oxidative modification in cultured HT22 mouse hippocampal cells. We found that chronic treatment with SSRI

antidepressant fluoxetine and SNRI antidepressant venlafaxine increased Trx and TrxR protein levels, but had no effect on Txnip protein levels in HT22 cells. TrxR can reduce Trx from an oxidized inactive form to a reduced active form, and maintains Trx reducing activity [137] . This findings suggest that antidepressants may increase Trx expression and also increase TrxR-facilitated Trx reduction. Previous studies have shown that antidepressants can increase antioxidant capacity. For example, it has been found that chronic treatment with fluoxetine reversed chronic stress-decreased total antioxidant capacity[138]. It has also been found that chronic treatment with venlafaxine at 5, 10 and 20 mg/kg (i.p.) for 21 days increased total antioxidant capacity in the rat hippocampus[139]. Chronic venlafaxine treatment was also found to increase total antioxidant capacity in mouse hippocampus [139]. Clinical studies also found that treatment with antidepressant sertraline, paroxetine or escitalopram for 12 weeks increased total antioxidant capacity in the serum of patients with the major depressive disorder as compared to controls [140]. Because Trx can reduce protein cysteine oxidation, scavenge ROS and inhibit oxidative stress; increased Trx and TrxR may contribute to antidepressant-upregulated total antioxidant capacity.

The possible roles of transcription factors Nrf2 and CREB in antidepressants-upregulated Trx and TrxR: Trx gene expression can be regulated by transcription factors nuclear factor E2-related factor 2 (Nrf2)[141]. Trx gene contains one ARE site in its promoter region while TrxR gene contains two ARE sites in its promoter region [141,142]. Nrf2 can bind to ARE sites and increase ARE-driven gene expression[143]. Studies have shown that Nrf2 can increase Trx and TrxR gene expression by targeting ARE sites. For

example, Kim and associates inserted Trx promoter region sequence with or without an ARE site into a luciferase reporter vector. Then this vector was cloned and transfected into human K562 cells. It has been found that luciferase activity was increased in cells transfected with Trx sequence with ARE site when compared to Trx sequence without ARE site. Further electrophoretic mobility shift assay also showed that Nrf2 antibody shifted protein-DNA binding band between nuclear proteins and ARE of Trx promoter [141] , suggesting that ARE site is important in basal activity of Trx gene promoter [141] . Overexpression of Nrf2 gene was found to enhance iron-containing porphyrin hemin-induced luciferase activity in cells transfected with Trx sequence containing ARE site, while dominant negative mutant of Nrf2 inhibited hemin-induced luciferase activity[141]. These findings suggest that Nrf2 can bind to Trx gene ARE site and induce ARE-driven Trx gene expression. Using electrophoretic mobility shift assay and chromatin immunoprecipitation assay, Nrf2 was found to bind to ARE site of TrxR gene promoter [142]. It has been reported that heavy metal cadmium increased TrxR promoter activity, but ARE deletion or mutation in TrxR promoter region can inhibit cadmium-activated Trx gene promoter activity [142]. Overexpression of Nrf2 gene was also found to enhance cadmium-induced TrxR promoter activity, but dominant negative mutant of Nrf2 inhibited TrxR promoter activity[142]. These findings suggest that that Nrf2 can bind to ARE sites of TrxR gene promoter regions and turn on TrxR gene expression. Since antidepressants increased Trx and TrxR protein levels, our findings also suggest that Nrf2 may mediate antidepressants-increased Trx and TrxR.

Indeed, a number of studies indicate that antidepressants can upregulate Nrf2 [42,144].

Chronic treatment with CORT for four weeks was found to reduce Nrf2 protein levels in mouse cerebral cortex and hippocampus, while treatment with antidepressant fluoxetine restored CORT-reduced Nrf2 protein levels [42]. Chronic unpredictable stress was also found to decrease nuclear protein levels of Nrf2, while antioxidant desipramine and duloxetine increased chronic unpredictable stress-reduced nuclear protein levels of Nrf2 in rat frontal cortex [144]. It has been found that antidepressants increase the protein levels of Nrf2 and induce antioxidant gene transcription [136] . Treatment with antidepressant desipramine increased nuclear Nrf2 protein levels in *MES23.5* cells, a dopaminergic neuronal cell line [136] . These studies further support that antidepressants may increase Nrf2 translocation from cytosol to the nuclear compartment, resulting in an increase of Trx and TrxR gene transcription.

Trx gene expression can also be regulated by transcription factor cAMP-responsive element binding protein (CREB) [145]. The promoter region of Trx gene contains a cAMP responsive element (CRE) [145] . CRE is primarily targeted by CREB. Once CREB is phosphorylated by protein kinases, phosphorylated CREB can translocate into the nucleus, bind to CRE sites and increase ARE-driven gene expression [146] . Previously it has been found that Trx mRNA and protein levels can be increased by nerve growth factor (NGF). NGF was also found to increase Trx gene promoter activity. Mutations of CRE in Trx gene promoter abolished NGF-activated Trx gene promoter activity [145]. Electrophoretic mobility shift assay also showed that NGF can increase the binding of nuclear protein to the CRE of Trx gene promoter and this CRE-nuclear protein binding band is shifted by CREB antibody[145], indicating that CREB can bind to CRE site of Trx

gene promoter. Many studies also showed that antidepressants increased CREB mRNA and protein levels, and CREB phosphorylation. For example, it has been found that chronic treatment with antidepressants imipramine, desipramine, sertraline, and fluoxetine for 21 days increased CREB mRNA levels in rat hippocampus[147]. Chronic fluoxetine treatment was also found to increase CREB protein levels in the mouse hippocampus, amygdale, cerebral cortex, hypothalamus, and thalamus [148] . Phosphorylated levels of nuclear CREB protein were also found to be increased by chronic treatment with antidepressants fluoxetine and reboxetine in the rat hippocampus [149]. It has been reported that treatment with antidepressants fluoxetine, sertraline, and paroxetine for 6 weeks increased nuclear-phosphorylated CREB levels and CRE-DNA binding activity in T-lymphocytes of MDD patients[150]. These studies suggest that chronic antidepressant administration can increase CREB expression and phosphorylation. These findings also suggest that CREB may contribute to antidepressants-induced upregulation of Trx.

Regulation of antidepressants on protein cysteine oxidation: Trx as a reductase can directly reduce cysteine oxidative modification such as protein sulfenylation and nitrosylation[151–153]. In the present study, we have found that chronic treatment with fluoxetine and venlafaxine inhibited H_2O_2-increased sulfenylated protein levels and GSNO-increased nitrosylated protein levels. These results suggest that fluoxetine and venlafaxine may upregulate Trx and further inhibit protein cysteine oxidation. Previously we found that chronic unpredictable stress increased protein cysteine sulfenylation and nitrosylation in mouse hippocampus and frontal cortex [98]. We also found that that chronic

treatment with stress hormone CORT increased total sulfenylated and nitrosylated proteins in primary cultured cerebral cortical cells and HT22 cells [119]. In the current study, we also found that fluoxetine and venlafaxine inhibited CORT-increased sulfenylated and nitrosylated proteins. Since chronic stress is a considerable important risk factor for depression and contributes significantly to depression development, inhibition of protein cysteine oxidation may play an important role in the pharmacological mechanism of antidepressants.

Trx can maintain peroxiredoxin at a reduced state and facilitate scavenging H_2O_2 and other peroxides[154,155]. Therefore antidepressant-increased Trx may further reduce H_2O_2 levels and inhibit lipid peroxidation process. Previously, treatment with fluoxetine at 10 mg/kg, i.p. was found to decrease stress-increased H_2O_2 levels in rat hippocampus and frontal cortex[156]. It was also found that fluoxetine treatment inhibited glutamate-increased ROS levels in mouse hippocampal slices, while venlafaxine treatment inhibited toxicant methylglyoxal-increased ROS levels in human brain microvascular endothelial cells [135,157]. Consistent evidence also indicates that antioxidants inhibit lipid peroxidation. For example, chronic treatment with fluoxetine for five weeks was found to inhibit lipid peroxidation induced by chronic unpredictable stress, stress hormone CORT treatment and endotoxin lipopolysaccharide treatment in mouse brain [138,149,158,159]. Chronic treatment with venlafaxine was also found to decrease stress-increased lipid peroxidation in mouse brain [139] . Clinical studies have also shown that treatment with SSRI and SNRI antidepressants imipramine, citalopram, sertraline, fluoxetine, venlafaxine, and escitalopram inhibited lipid peroxidation products F2-isoprostanes and MDA levels in the

serum of patients with MDD [70,160]. These findings, together with our results suggest that antidepressants may upregulate Trx, subsequently facilitating scavenge of ROS, and inhibit protein cysteine oxidation and lipid peroxidation, which contributes to protective effects of antidepressants against oxidative stress.

Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to L-citrulline, which produces nitric oxide (NO)[161]. There are three major isoforms of NOS such as neuronal (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed in neuronal and endothelial cells respectively, while iNOS is an inducible form that is involved in immune response [161]. Many findings indicate that antidepressants can inhibit NOS and reduce NO production. For example, it has been found that chronic treatment with venlafaxine treatment at 5 and 10 mg/kg i.p. for seven days reduced NO levels in mice brain as compared to controls[162]. It has been found that antidepressants sertraline, fluvoxamine and fluoxetine at both 10 and 20 mg/kg (s.c.) for two days prior to LPS injection decreased the nitric oxide level in LPS treated mice[163]. Imipramine at 15 mg/kg and venlafaxine at 6 mg/kg i.p. decreased nitrite and nitrate levels in mice[164]. It has been found that treatment with fluoxetine at 10 mg/kg (i.p.) for 1, 3 and 7 days decreased the nNOS protein levels in the mice hippocampus [165]. It has been reported that antidepressants imipramine, citalopram, and paroxetine at 100μM concentration reduced the NOS activity by measuring the amount of [^{3}H] L-citrulline in vitro in the rat hippocampus [166] . Mice exposed to chronic unpredictable mild stress for five weeks showed increased iNOS protein levels, while pretreatment with sertraline at 10 mg/kg orally for 35 days reduced stress-increased iNOS protein levels in mouse

brain[167]. Sertraline was also found to inhibit endotoxin LPS-increased iNOS protein levels in cultured rat BV2 microglia cells[81]. eNOS mRNA levels were also found to be decreased by chronic treatment with antidepressants paroxetine at 10 mg/kg (i.p.), milnacipran at 30 mg/kg (i.p.) and mirtazapine at10 mg/kg (i.p.) for three weeks in the cerebellum, frontal cortex, thalamus, and striatum[168]. These studies suggest that antioxidants may upregulate NOS and increase NO production. Since H_2O_2 and NO can attack protein cysteine residues and cause protein cysteine sulfenylation and nitrosylation, antioxidants may inhibit protein cysteine oxidation by not only upregulating Trx but also downregulating H_2O_2 and NO.

***Regulation of antidepressants on apoptosis signal-regulating kinase 1* (*ASK1) signaling*:** Trx can bind to ASK1 and inhibit ASK1 activation. ASK1 is a stress-responsive mitogen-activated protein kinase kinase kinase and plays an important role in regulating neuronal differentiation, apoptosis, and inflammation[169]. Trx can bind to ASK1 and maintain ASK1 in an inactive state. Once cells are insulted by ROS, cytokines and other stressors, ASK1 dissociates from Trx, resulting in ASK1 autophosphorylation, subsequently inducing phosphorylation of c-Jun N-terminal kinase (JNK) and p38 kinase, and activating JNK/P38 activated signaling pathways[154,155,170]. It has been found that chronic treatment with fluoxetine decreased CORT-increased phosphorylated JNK levels [171]. Chronic fluoxetine treatment has also been found to reduce phosphorylated p38 levels in mouse hippocampus[172]. Venlafaxine has also been found to inhibit methylglyoxal-induced JNK phosphorylation in human brain microvascular endothelial cells[157]. Antidepressants trazodone and imipramine were also found to inhibit prenatal

stress-induced JNK and p38 phosphorylation in rat hippocampus and frontal cortex[173]. These studies suggest that antidepressants may upregulate Trx and inhibit ASK1, further reducing JNK and p38 MAP kinase phosphorylation. The role of ASK1/JNK/p38 signaling in pharmacological treatment requires further investigation.

In summary, chronic treatment with SSRI antidepressant fluoxetine and SNRI antioxidant venlafaxine increased Trx and TrxR protein levels in HT22 mouse hippocampal cells. Treatment with fluoxetine and venlafaxine also inhibited H_2O_2- and CORT-increased sulfenylated protein levels, and inhibited NO donor GSNO- and CORT-increased nitrosylated protein levels. Since Trx can reverse protein cysteine sulfenylation and nitrosylation, these results suggest that antioxidants may inhibit protein cysteine oxidation by upregulating Trx. Our findings also indicate that upregulation of Trx may contribute to the protective effect of antidepressants against oxidative stress and further indicate that Trx is a potential therapeutic target for the treatment of depression.

4.4. The effect of glucocorticoid on NLRP3 inflammasome in microglia cells:

Upregulation of Txnip by CORT in mouse microglia: Microglia contributes to immune defense in the brain and play significant roles in the regulation of inflammation. Activation of microglia generates ROS and NO that promote oxidative stress. Since Trx system also plays a role against inflammation and oxidative stress; in the present study, we also analyzed the effect of CORT on Trx, TrxR, and Txnip in primary cultured mouse microglia and N9 mouse microglial cells. We found that although chronic CORT treatment had no effect on Trx and TrxR protein levels, this treatment upregulated Txnip

protein levels in both primary cultured mouse microglia and N9 cells. This finding suggests that chronic CORT treatment may also cause oxidative stress not only in neurons but also in microglia.

Regulation of CORT on NLRP3 inflammasome signaling: Txnip can interact with the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) and activate the NLRP3 inflammasome. Many studies using co-immunoprecipitation have shown that Txnip can directly bind to NLRP3 [174,175]. Binding of Txnip to NLRP3 can induce coupling of the components of NLRP3 inflammasome. The NLRP3 protein contains three domains namely C-terminal leucine-rich repeat domain (LRR), a central nucleotide binding and oligomerization domain (NACHT), and an N-terminal pyrin domain (PYD). The LRR domain recognizes the ligands, and mediates the protein-protein interaction; the NACHT facilitates inflammasome formation, and PYD enables the interaction between NLRP3 and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). NLRP3 inflammasome consists of NLRP3 protein, ASC and procaspase-1[176]. It has been found that treatment with L-homocysteine not only increased Txnip and NLRP3 co-localization but also increased NLRP3 and ASC co-localization in kidney podocytes, while Txnip siRNA inhibited NLRP3 and ASC co-localization[177]. Mice with the condition of hyperhomocysteinemia also showed increased NLRP3 and Txnip co-localization, and NLRP3 and ASC co-localization in glomeruli, while *in-vivo* knock out of Txnip showed a decreased NLRP3 and ASC co-localization[177]. These findings suggest that Txnip-NLRP3 binding may facilitate NLRP3 inflammasome assembly. In the nervous system, NLRP3 is

mainly expressed in glia[178]. We found that chronic CORT treatment increased Txnip-NLRP3 binding in N9 microglia cells. We also found that Txnip-NLRP3 binding activity was increased in the hippocampus and frontal cortex of mice subjected to chronic unpredictable stress when compared to controls. Previously chronic unpredictable mild stress has been found to increase NLRP3-ASC binding in rat hippocampus[179]. mRNA levels of NLRP3 have been found to be increased by chronic CORT treatment in cultured rodent astrocyte and microglia[180,181]. These findings suggest that chronic stress-increased CORT may further increase Txnip levels and promote Txnip-NLRP3 binding and facilitate NLRP3 inflammasome assembly.

Txnip-induced NLRP3 inflammasome activation can cleave procaspase-1 and activate caspase-1, converting pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, which accelerate pro-inflammatory responses[182]. It has been found that treatment with NLRP3 inflammasome inducer monosodium urate (MSU) increased Txnip protein levels, Txnip/NLRP3 binding activity and IL-1β release in human THP-1 monocyte cells, while knocking down Txnip by Txnip siRNA reduced MSU-induced IL-1β release[175]. Caspase-1 activation and IL-1β release were found to be decreased in macrophage of Txnip knock out mice when compared to wild-type mouse[183]. It has also been reported that treatment with a high concentration of glucose (30 mM) increased Txnip protein levels, Txnip and NLRP3 co-localization, procaspase-1 cleavage, and IL-1β and IL-18 release; while Txnip shRNA inhibited glucose-induced IL-1β and IL-18 release in cultured U937 human monocytes, human retinal microvascular endothelial cells, and human kidney HK-2 cells [174,184,185]. These findings support that Txnip can bind to NLRP3, subsequently resulting in

activation of caspase-1 and release of IL-1β and IL-18.

In the present study, we found that chronic CORT treatment reduced procaspase-1 levels and increase caspase-1 enzyme activity. Our findings suggest that CORT may induce cleavage of procaspase-1 and release of mature caspase-1, subsequently inducing activation of caspase-1. Further, we also found that chronic CORT treatment increased IL-1β levels in the culture medium, suggesting that CORT may increase IL-1β secretion. Our findings suggest that CORT-upregulated Txnip may activate NLRP3 inflammasome, resulting in caspase-1 activation and IL-1 β secretion. Previously it has been reported that chronic unpredictable stress-induced procaspase-1 cleavage, and increased IL-1β and IL-18 protein levels in mouse hippocampus [186,187]. It has also been found that chronic restraint stress increased the protein levels of p20 subunit, a cleaved product of procaspase-1 and protein levels of IL-1β in mouse hippocampus [188,189] . Chronic restraint stress was also found to increase mRNA and protein levels of IL-1β and IL-18 in rat prefrontal cortex and hippocampus [186]. Chronic CORT treatment was also found to increase caspase-1 activity, and IL-1β and IL-18 protein levels in cultured rodent astrocyte and microglia [180,181]. These findings together suggest that chronic stress increase glucocorticoids, resulting in upregulation of Txnip and activation of the NLRP3 inflammasome, subsequently activating caspase-1 and releasing IL-1β and IL-18 in rodents.

The role of Txnip in CORT-regulated NLRP3 inflammasome signaling: To elucidate the role of Txnip in CORT-induced NLRP3 inflammasome activation, we analyzed the

effect of Txnip knockout on chronic CORT treatment-increased caspase-1 activity and IL-1β secretion. Txnip gene was knocked out by Txnip sgRNA/Cas9 in N9 microglia cells. We found that although chronic CORT treatment increased caspase-1 activity and increased the supernatant IL-1β levels in scrambled sequence transfected N9 cells, this treatment has no effect on caspase-1 activity and medium IL-1β levels in Txnip sgRNA transfected N9 cells. These results suggest that Txnip mediates CORT-induced caspase-1 activation and IL-1β release. These findings together suggest that CORT-increased Txnip may bind to NLRP3 and facilitate formation of NLRP3 inflammasome, resulting in procaspase-1 cleavage, caspase-1 activation, and IL-1β release. These findings also suggest that Txnip mediates chronic CORT promoted the inflammatory process.

In summary, we found that prolonged treatment with stress hormone CORT increased Txnip protein levels in mouse microglia cells. CORT treatment also increased Txnip-NLRP3 interaction in cultured microglia cells and mouse brain. Further, we found that chronic CORT treatment increased procaspase-1 cleavage, activated caspase-1 and released IL-1 β and that knocking out Txnip inhibited CORT-induced caspase-1 activation and IL-1release. Our findings suggest that chronic CORT treatment may upregulate Txnip and increase Txnip-NLRP3 binding, facilitating NLRP3 inflammasome formation, which promotes procaspase-1 cleavage, activates caspase-1 and releases IL-1β. Our findings also suggest that Txnip-activated NLRP3 inflammasome signaling may mediate CORT-induced inflammatory process.

CHAPTER 5: Summary, significance, limitation, and future studies

5.1. Summary

First, we found that chronic CORT treatment in mouse hippocampal HT22 cells and primary cultured cerebral cortical neurons increased Txnip protein levels, but had no effect on Trx and TrxR protein levels. Treatment with GR antagonist RU-486 can reduce CORT-increased protein levels of Txnip in both HT22 and primary cerebral cortical neuronal cells. We have also found that chronic treatment with CORT increased the protein cysteine sulfenylation and nitrosylation in HT22 cells. Knocking out Txnip inhibited protein sulfenylation and nitrosylation induced by CORT. As shown in **Fig. 27**, these findings suggest that chronic CORT treatment can upregulate Txnip in neuronal cells, resulting in Trx inhibition that facilitates protein cysteine oxidative modification, which contributes to stress-induced oxidative stress process.

Second, we found that chronic treatment of antidepressants fluoxetine and venlafaxine increased Trx and TrxR protein levels, but has no effect on Txnip protein levels in HT22 cells. Chronic treatment with fluoxetine and venlafaxine not only inhibited H_2O_2-induced protein cysteine sulfenylation and NO donor GSNO-induced protein cysteine nitrosylation; but also inhibited CORT-induced protein cysteine sulfenylation and nitrosylation. Since Trx can reverse protein cysteine oxidative modification, our results suggest that antidepressants may upregulate Trx, further inhibiting cysteine oxidative modification (**Fig. 27)**.

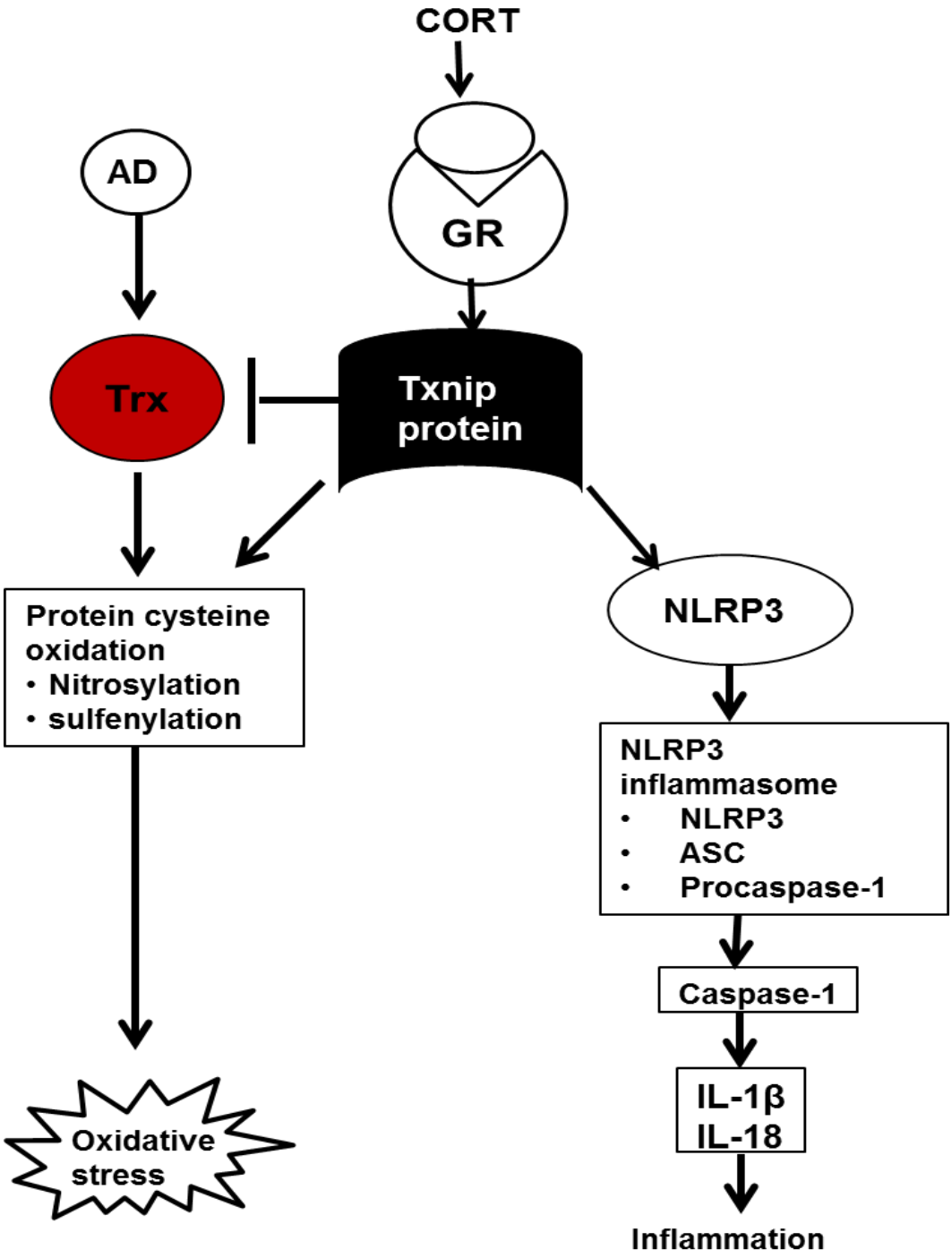


Fig. 27. Summary: Possible role of Thioredoxin interacting protein in corticosterone induced oxidative stress and inflammation processes. CORT**:** corticosterone, Txnip: thioredoxin interacting protein, AD: antidepressants, Trx: thioredoxin, NLRP3: nod-like receptor protein 3, ASC: Apoptosis-associated speck like protein, IL-1β: Interleukin-1β, IL-18: Interleukin 18

Third, we found that chronic CORT treatment increased protein levels of Txnip, but had no effect on protein levels of Trx and TrxR in mouse N9 microglia and primary cultured microglia. Chronic CORT treatment also increased Txnip-NLRP3 binding activity in N9 cells. Txnip-NLRP3 binding activity was also increased in frontal cortex and hippocampus of mice exposed to chronic unpredictable stress when compared to controls. We also found that chronic CORT treatment increased procaspase-1 cleavage, caspase-1 activity and IL-1β release in N9 cells. Knocking out Txnip inhibited CORT-increased caspase-1 activity and IL-1β release in N9 cells. As shown in **Fig. 27**, our findings suggest that chronic CORT treatment can upregulate Txnip and increase Txnip-NLRP3 protein interaction in microglia, further activating NLRP3 inflammasome, resulting in activation of caspase-1 that releases mature IL-1β, which facilitate the inflammatory process.

5.2. Significance

Chronic stress contributes significantly to the pathophysiological development of depression and other psychiatric disorders. Chronic CORT treatment and chronic stress can cause oxidative stress and inflammation. As discussed in the introduction section, many studies have shown that oxidative stress and inflammation play an important role in depression development and progression[64,69,73,77]. Since Txnip is an endogenous inhibitor for Trx and can amplify oxidative stress and facilitate inflammation; Txnip and Trx may be a potential target for the treatment of depression and other psychiatric disorders.

5.3. Limitations and future studies

In the present study, there are several limitations. First, our results show that corticosterone treatment for 5 days increased Txnip mRNA and protein levels in HT22 cells. We have also shown that chronic corticosterone treatment didn't increase Trx protein levels. Although our results suggest that increased levels of Txnip protein in HT22 cells may bind to Trx and inhibit Trx activity, we didn't measure Trx activity or Trx-Txnip binding. In the future, Trx activity and Trx-Txnip binding by redox western blot will be analyzed.

Second, various brain regions and cell types respond to stress. However, we only measured the effect of CORT in Txnip protein levels in primary cultured mouse cerebral cortical neurons, hippocampus-derived neuronal cell line, primary microglia and microglia derived cell line. In future, the effect of CORT on protein levels of Txnip will be measured in other brain regions that process the emotional thoughts such as amygdala and nucleus accumbens and in other cell types that respond to stress such as astrocytes and oligodendrocytes etc.

Third, our work was restricted to the in-vitro model. Further animal stress model involving Txnip knockdown in animal brain should be performed to determine if Txnip is involved in stress-induced depressive-like behaviour.

Fourth, although we have shown that chronic CORT treatment increased protein sulfenylation and nitrosylation, we didn't analyze which cysteine amino acids are

modified. In the future, this work should be extended to determine the exact amino acid sequence modification to understand which cysteine modifications take place due to stress hormone treatment. In the future, the MDD patients' samples should be used to determine the correlation between neurocognitive or emotional defects and protein oxidative modifications.

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